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Keynote Symposium (1)

1

**Stem Cells, Pluripotency and Nuclear Reprogramming.**
*R. Jaenisch; Whitehead Institute for Biomedical Research, Cambridge, MA*

One of the key issues raised by nuclear cloning is the question of genomic reprogramming, i.e. the mechanism of resetting the epigenetic modifications that are characteristics of the adult donor nucleus to ones that are appropriate for an embryonic cell. The mechanisms by which embryonic stem (ES) cells self-renew while maintaining the ability to differentiate into virtually all adult cell types are not well understood. Major progress has been achieved to understand the molecular circuitry of pluripotency and self-renewal. This information provides crucial insights into mechanisms by which pluripotent cells may be stimulated to differentiate into different cell types or by which somatic cells might be reprogrammed back to the pluripotent state by exposure of the somatic nucleus to the egg cytoplasm. The recent demonstration of in vitro reprogramming using transduction of 4 transcription factors by Yamanaka and colleagues represents a major advance in the field. Major questions regarding the mechanism of in vitro reprogramming need to be understood and will be one focus of the talk. Also, our progress in using iPS cells for therapy and for the study of complex human will be summarized.
Minisymposium 1: Cancer Cells (2 – 7)

2 Imaging the Metastatic Process.
E. Sahai, S. Giampieri; Tumour Cell Biology Laboratory, Cancer Research UK London Research Institute, London, United Kingdom

Cancer cells can invade surrounding tissue either as single cells or in collective units. We use intravital imaging to demonstrate a reversible transition to a motile state as breast cancer cells spread. Imaging primary tumours reveals heterogeneity in cell morphology and motility. Two distinct modes of motility are observed: collective and single-celled. By monitoring the localisation of Smad2 and the activity of a TGFβ-dependent reporter gene during breast cancer cell dissemination we demonstrate that TGFβ signalling is transiently and locally activated in motile single cells. TGFβ1 switches cells from cohesive to single cell motility through a transcriptional programme involving Smad4, EGFR, Nedd9, and numerous regulators of actomyosin contraction: M-RIP, FARP and RhoC. In contrast, different regulators of the actomyosin cytoskeleton are used during collective invasion. Blockade of TGFβ signalling prevents cells moving singly in vivo but does not inhibit cells moving collectively. Cells restricted to collective invasion are capable of lymphatic invasion but not blood-borne metastasis. Constitutive TGFβ signalling promotes single cell motility and intravasation but reduces subsequent growth in the lungs. Thus, transient TGFβ signalling is optimal for blood-borne metastasis.

3 Regulation of Cellular Self-Renewal by the Arf Tumor Suppressor.
A. Gromley, M. L. Churchman, C. J. Sherr; Department of Tumor Cell Biology, St. Jude Children’s Research Hospital/HHMI, Memphis, TN

The p19Arf and p16Ink4a tumor suppressor proteins (encoded by alternative reading frames of the Cdkn2a (Ink4a-Arf) locus) respectively regulate p53 and Rb-dependent gene expression programs triggered by aberrant oncogenic signals. When expressed at elevated levels, p19Arf and p16Ink4a induce cell cycle arrest, senescence, and/or apoptosis to eliminate the expansion of incipient tumor cells. However, the fact that the Ink4a-Arf locus is conserved in species of mammals and birds that do not develop cancer begs the question of whether these “tumor suppressors” play broader physiologic roles in governing cellular self-renewal. Emerging findings now suggest that the Ink4a-Arf locus regulates the homeostatic equilibrium between self-renewing tissue stem cells and their amplifying progeny in several different biological settings. For example, recent use of lineage tracing strategies in mice has documented transient expression of Arf in proliferating progenitor cells during specific stages of male germ cell development, formation of stratified epithelia, and during postnatal regression of the hyaloid vasculature system in the vitreous of the eye. As a general rule, the Ink4a-Arf locus is epigenetically silenced in stem cells but becomes poised to respond to proliferative stress signals as cells undergo lineage commitment and further differentiation. Disruption of Ink4a-Arf gene silencing and the ensuing activation of the locus limit cellular self-renewal and contribute to aging phenomena; conversely, deletion of the locus strongly predisposes to aberrant cellular proliferation and tumor formation. This fundamental role of the Ink4a-Arf locus in gating cellular self-renewal appears to explain both its evolutionary conservation and broad impact on processes governing both aging and cancer.

4 The Role of miR-128 in Glioma Tumor Initiation and Maintenance.
T. Papagiannakopoulos1, A. Weaver2, R. Gill1, I. Hernandez1, E. Huillard3, D. H. Rowitch2, F. M. White2, K. S. Kosik1; 1Neuroscience Research Institute, UC Santa Barbara, Santa Barbara, CA, 2The David H. Koch Institute for Integrative Cancer Research, MIT, Cambridge, MA, 3Pediatrics and Neurological Surgery and the Institute for Regeneration Medicine, UC San Francisco, San Francisco, CA
MicroRNAs (miRNAs) are a class of small non-coding RNAs (20-25nt) that bind to the 3' untranslated region of target mRNAs through an imperfect match to repress their translation and stability. miRNAs regulate diverse cellular functions in various biological processes. Several studies have confirmed that miRNAs regulate cancer cell proliferation and apoptosis. Furthermore, these non-coding RNAs can control cellular identity and mediate differentiation. Aberrant expression of miRNA genes can lead to human diseases, including cancer. Gliomas are highly aggressive brain tumors that are thought to arise from malignant Neural Stem Cells (NSCs) that have lost their differentiation potential. To investigate the function of miRNAs in gliomagenesis, we chose an established glioma mouse model. This model utilizes tumor-initiating NSCs (tiNSCs) that contain known glioma lesions. To determine whether miRNAs are involved in the transformed state of these tiNSCs, we profiled 187 mouse miRNAs and identified numerous repressed miRNAs. Among these miRNAs, miR-128 was down-regulated. This miRNA has been previously implicated in CNS development and is down-regulated in human glioma tumors. Thus, with a combination of molecular, cellular and in vivo methods we proceeded to characterize the role of miR-128 in NSC-induced gliomagenesis and glioma tumor maintenance. In culture, miR-128 overexpression led to repression of tiNSC growth and differentiation into a neuronal lineage. MiR-128 represses growth by directly targeting a network of key glioma oncogenes involved in Receptor Tyrosine Kinase (RTK) mitogenic signaling. With the use of quantitative tyrosine phospho-proteomics we are able to determine the extent of miR-128 regulation on tyrosine signaling in tiNSCs. We are currently testing the potential of miR-128 to repress tiNSC gliomagenesis and tumor maintenance in vivo. Using a novel peptide-based therapeutic approach we are attempting to suppress tumor growth by overexpressing miR-128 in glioma tumors by systemic delivery. Overall, using a multifaceted approach we are able to characterize the tumor suppressive role of miR-128 in gliomas.

5  
**p21WAF1 Dependent Emi1 Down-regulation After DNA Damage Maintains G2 Arrest.**  
J. Lee1,2, J. A. Kim1,2, V. Barbier2, A. Fotedar2, R. Fotedar1,2,3; 1Institut de Biologie Structure Jean Pierre Ebel, Grenoble, France, 2Sidney Kimmel Cancer Center, San Diego, CA, 3Burnham Institute for Medical Research, La Jolla, CA

Cell cycle checkpoints safeguard genome integrity. p21WAF1, an inhibitor of cyclin dependent kinases (CDKs) has an important role in checkpoint response to DNA damage. It has been previously shown that following DNA damage, p21<sup>+/+</sup> cells stably arrest in G2 whereas p21<sup>−/−</sup> cells ultimately progress into mitosis. We have analyzed the role of p21WAF1 in G2-M phase checkpoint control and in prevention of polyploidy after DNA damage. The role of Anaphase promoting complex (APC), whose activity is not high in G2 phase, has not been extensively studied in the DNA damage checkpoint in mammalian cells. APC, a multi-protein complex with E3-ubiquitin ligase activity, regulates progression through mitosis to G1 and its substrates include cyclins (A2 and B1). We report that APC activation in G2 leads to stable arrest in irradiated p21<sup>+/+</sup> cells. We find that p21 down-regulates Emi1, an APC inhibitor whose destruction controls progression through mitosis to G1, in cells arrested in G2 by DNA damage. This down-regulation contributes to APC activation and results in the degradation of key mitotic proteins including cyclins A2 and B1 in p21<sup>+/+</sup> cells that prevents the G2 arrested cells from entering mitosis. Inactivation of APC in can overcome the G2 arrest in irradiated p21<sup>−/−</sup> cells. Short interference RNA mediated Emi1 down-regulation prevents irradiated p21<sup>−/−</sup> cells from entering mitosis whereas concomitant inactivation of APC counteracts this effect. Our results demonstrate that Emi1 down-regulation and APC activation leads to stable p21 dependent G2 arrest after DNA damage. Our work is the first demonstration that Emi1 regulation plays a role in the G2 DNA damage checkpoint. Further, our work identifies a new p21 dependent mechanism to maintain G2 arrest after DNA damage.

6  
**Effects of p120 Ablation on Normal Mammary Gland Development and PyMT-Induced Mammary Tumor Progression.**  
S. Kurley1, M. A. Davis1, B. Bierie1, W. J. Muller2, A. B. Reynolds1; 1Cancer Biology, Vanderbilt University, Nashville, TN, 2Biochemistry and Medicine, McGill University, Montreal, QC, Canada
p120-catenin (p120) modulates epithelial cell-cell adhesion by controlling the stability of E-cadherin, an important suppressor of tumor progression and metastasis. Areas of p120 loss are observed in most carcinoma types, including ~10% of ductal breast carcinomas, but the significance of this observation is unclear. Here, we have used tissue specific p120 knockout mice to examine the in vivo effects of p120 ablation in normal and transformed mammary epithelium. Surprisingly, p120 appears to be essential for mammary gland development. Cre expression by the MMTV promoter in our model is initiated concurrently with pubertal mammary gland development. Under this condition, p120 null cells were clearly evident in developing ducts at 4 weeks, but were undetectable at time points thereafter. Ductal outgrowth was initially delayed as p120 null cells were eliminated and replaced by p120 positive cells that subsequently behaved normally. Furthermore, at early time points, p120 knockout mice presented stunted growth of mammary terminal end buds that was overcome at subsequent time points. Exactly why p120 is required is not yet clear, but p120 null cells were also deficient in E-cadherin, as well as β-catenin, which could account for the observed changes to tissue morphology, apparently compromised cell-cell adhesion, and drastic alterations in mammary gland development. To examine p120 loss in the context of tumor progression, we crossed our p120 knockout mice into the MMTV-Polyoma Middle T (PyMT) mouse model of breast cancer described previously by Dr. W. Muller. In the context of PyMT, p120 ablated cells survived and participated in tumor formation. Interestingly, p120 null tumor regions were exclusively pseudopapillary, whereas both solid and pseudopapillary tumors were observed when p120 was present. p120 negativity was also associated with macrophage infiltration, an increased presence of myofibroblasts, and almost complete absence of E-cadherin and junctional β-catenin. Tumor latency and volume in p120 knockout mice were unchanged, however lung metastases were significantly increased. Experiments are underway in both systems to clarify underlying mechanisms.

7 Pseudopodial-Enriched Atypical Kinase One (PEAK1) is a Novel Protein that Regulates ErbB2-Induced Cellular Mitogenesis and Migration.

J. A. Kelber1,2, Y. Wang1,2, H. Tran Cao3, W. Wang1,2, S. Kaushal2, R. Hoffman3, M. Bouvet2,3, R. Klemke1,2; 1Pathology, University of California San Diego, La Jolla, CA, 2Moores Cancer Center, University of California San Diego, La Jolla, CA, 3Department of Surgery, University of California San Diego, La Jolla, CA

Cell migration is an integral process during development, immune function, and cancer metastasis. Using quantitative phosphoproteomics to analyze migratory regions within the cell known as pseudopodia, we recently identified a new phosphoprotein we’ve termed pseudopodial-enriched atypical kinase one (PEAK1). We have found that PEAK1 is necessary for the mitogenic and migratory effects of the epidermal growth factor (EGF) receptor, ErbB2, in cancer cells. Additionally, PEAK1 undergoes Src-dependent tyrosine phosphorylation in response to EGF ligands and extracellular-matrix (ECM) proteins; potentiates fibronectin-induced Paxillin, ERK and CAS phosphorylation; binds to ErbB2, Raf, CAS and Crk and co-localizes with the actin and focal adhesion cytoskeleton. Based upon our functional and biochemical analysis of PEAK1, we predicted that PEAK1 may mediate ErbB2-induced tumorigenesis by coupling ErbB2 activation to the oncogenic Ras/Raf/ERK and Src/CAS/Crk signaling cascades. In this regard, we show that PEAK1 binds to ErbB2. Using PEAK1 truncation mutants that bind ErbB2 and CAS or ErbB2, CAS and Crk, we further demonstrate that PEAK1 mediates ErbB2-induced cell migration/invasion via the Src/CAS/Crk pathway. Finally, we show that PEAK1 is promotes oncogenic growth in vitro and in vivo and is upregulated in metastatic cancer cells. Collectively, these studies provide a mechanistic understanding of how PEAK1, an important regulator of cancer progression, couples to the cytoskeletal signaling machinery in order to mediate cancer cell migration/invasion. With this new understanding of the mechanism of PEAK1 function, unique small molecule reagents may be developed to target specific PEAK1-mediated functions of ErbB2 during cancer progression.
**Minisymposium 2: Cell-Cell Interactions (8 – 13)**

8

**Drosophila Lar, a Receptor Tyrosine Phosphatase, Regulates Cell Adhesion between Germline Stem Cells and the Niche.**

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Adult stem cells function to replenish the pool of highly differentiated but short-lived cells in tissues. Adult stem cells can self-renew to maintain their numbers and differentiate to give rise to tissue cells. Many adult stem cells reside in a niche, which provides signals to critically balance the choice between self-renewal and differentiation. Stem cell-niche cell adhesion is important to regulate stem cell fate by keeping stem cells close enough to receive short-range signals. Stem cells can attach to and orient towards the niche to achieve asymmetric stem cell divisions. We hypothesize that one outcome of the niche signal important for stem cell maintenance might be to increase the cell adhesion between stem cells and the niche. We examined the role of cell adhesion in specifying stem cell fate using the *Drosophila* male germline as a model system. 

**leukocyte antigen receptor** (*lar*) was identified as being upregulated in an expression analysis of early germ cells. *lar* is required in male germ cells for maintenance of germline stem cells (GSCs); male germ cells mutant for *lar* are lost to differentiation. Wild-type male GSCs anchor to the niche through adherens junctions. Ultrastructural analyses reveal that the adherens junctions between GSCs and the niche are weak in *lar* mutants suggesting a role for Lar in maintaining GSCs by promoting niche-stem cell adhesion. Lar is expressed by GSCs and localizes to the niche-GSC interface consistent with its role in regulating adhesion between the niche and GSCs. Germ cells expressing Lar may recognize the niche via the heparosulfate proteoglycan Dally-like (Dlp), a known ligand of Lar, which is expressed by the niche cells. Lar also interacts with Enabled (Ena), a member of the Ena/VASP family of proteins that regulate F-actin elongation. Ena is expressed in GSCs and localizes to the hub-GSC interface. This polarized localization of Ena is lost in *lar* mutants. Our current model based on these data is that Lar through its interaction with Dlp ensures the activation and/or correct localization of Ena to the hub-GSC interface to promote hub-GSC adhesion by localized regulation of cortical F-actin assembly.

9

**Calcium-Dependent Dynamics of Cadherin Interactions at Synapses and Cell-Cell Junctions.**

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Cadherins, a family of homophilic adhesion proteins, play an important role in the calcium-dependent dynamics of structural and synaptic plasticity along with cell-cell contact formation and remodeling. A unique feature of cadherin-cadherin interactions is a strong dependence on extracellular Ca²⁺—which serves to rigidify the molecular structure of cadherins and promote trans-junctional interactions. Fluctuations in extracellular Ca²⁺ could thus, in principle, modify the dynamics of cadherin homophilic interactions across junctions, but this remains unexplored. We have developed a genetically encoded Förster Resonance Energy Transfer (FRET) reporter system that permits the direct visualization and quantification of spatiotemporal dynamics of N-cadherin interactions across intercellular junctions in live cells. We found that upon rapid chelation of extracellular Ca²⁺ N-cadherin exhibited a sudden, but partial, loss of homophilic interactions. A cadherin mutant that lacks adhesive activity (W2A) exhibited a more substantial loss of homophilic interactions, suggesting two types of cadherin interactions—one that is rapidly modulated by changes in extracellular Ca²⁺ and another with relatively stable adhesive activity that is Ca²⁺-independent. These data indicate a previously unrecognized sensitivity of cadherin molecules to fluctuations in extracellular Ca²⁺. To transfer our FRET reporter system into neurons, N-cadherin constructs are expressed using viral gene delivery such that a FRET donor
(cerulean-cadherin) is expressed in presynaptic neurons while a FRET acceptor (venus-cadherin) is expressed in postsynaptic neurons. FRET will then be used to detect cadherin interactions between pre- and postsynaptic cells under various conditions. In particular, we will examine the effects of synaptic activity and varying extracellular calcium concentrations on cadherin-cadherin dynamics. These experiments will test our hypothesis that cadherins act as extracellular calcium detection system to coordinate synaptic plasticity across the synapse.

10 Imaging Trans-Synaptic Protein-Protein Interactions to Study the Molecular Mechanisms of Synapse Development.
A. Thyagarajan, A. Y. Ting; Chemistry, Massachusetts Institute of Technology, Cambridge, MA

Methods to image the size and dynamic properties of inter-cellular protein-protein interactions (PPIs) will facilitate studies of cell-cell interactions in development and in disease. Here we introduce a method for fluorescent labeling of inter-cellular PPIs via proximity biotinylation between a protein fusion to biotin ligase (BirA), and a protein fusion to BirA’s 15-amino acid “acceptor peptide” (AP) substrate. When the two proteins interact BirA covalently ligates a biotin onto the acceptor peptide. Biotinylated protein complexes are visualized by staining with fluorophore-conjugated monovalent streptavidin. We applied this methodology to image the trans-synaptic neurexin-neuroligin adhesion complex at single neuronal synapses in hippocampal neurons. We demonstrate that this trans-synaptic adhesion complex grows both during developmental maturation of synapses and in response to acute chemical stimulation. Complex growth occurs via a combination of new neurexin-neuroligin interaction formation, and arrest of both neurexin and neuroligin internalization. Strikingly, both processes require NMDA receptor activity. Using proximity biotinylation in conjunction with pHluorin-GluR1 time-lapse imaging, we discovered that neurexin-neuroligin complex growth is required for activity-dependent AMPA receptor recruitment to the postsynaptic membrane. Together, our results suggest an important role for the neurexin-neuroligin interaction in synapse maturation and stabilization.

11 Mechanosensitive Cadherin Complex with Links to the Keratin Cytoskeleton Regulates Cell Polarity and Directed Protrusions Driving Collective Cell Migration.
G. Weber, M. A. Bjerke, D. W. DeSimone; Department of Cell Biology, University of Virginia, Charlottesville, VA

The mesendoderm of the Xenopus gastrula is a motile population of cells comprising a multilayered cell sheet that moves directionally upon a fibronectin (FN) matrix assembled by the blastocoel roof. The leading edge protrusions of each cell within the sheet are in contact with FN while the rear or “retracting” edge of each cell rests upon the leading edge of the cell behind it. The fidelity of this morphogenetic process requires cell-cell contact; directed monopolar protrusive activity characteristic of this collective behavior is lost upon dissociation to single cells. Moreover, the intact sheet advances under tension and release of substrate adhesion with a mAb directed against integrin α5β1 causes the rapid retraction of the sheet. We hypothesize that anisotropic tension transmitted through C-cadherin (C-cad) adhesions is an intrinsic property of this tissue required for the coordination of directed cell motility. To approach this problem we developed a magnetic bead pull assay and asked whether mechanical force applied through cadherins could re-establish cell polarity and directed protrusive activity in single dissociated mesendoderm cells on FN. Mechanical force applied to attached C-cadFc coated beads but not FN or poly-l-lysine coated beads induced multipolar cells to become monopolar protrusive and to migrate away from the direction of magnetic bead pull. Co-immunoprecipitation and colocalization analyses in live explants established that a subset of C-cad molecules in these cells was associated with the keratin intermediate filament network through the catenin family member plakoglobin. Dissociated mesendoderm cells obtained from cytokeratin or plakoglobin antisense morpholino-injected embryos were unresponsive to C-cad bead pull. Intact mesendoderm from morphant embryos had defects in cell polarity and cytoskeletal organization. We have identified a cadherin mechanosensory complex that specifies cell polarity in a true collective cell migration
event. Linkage of classical cadherins to the keratin filament network is critical for mechanosensitive signaling to direct cell polarity and movement in this tissue. This work was supported by USPHS grants HD26402 and GM83542.

12 Alpha-Catenin Regulation of Actin Dynamics and Cell-Cell Adhesion.
A. V. Kwiatkowski1,2, J. M. Benjamin2,1, S. Pokutta3,1, W. I. Weis3,1, W. Nelson2,1; 1Dept. of Molecular and Cellular Physiology, Stanford University, Stanford, CA, 2Dept. of Biology, Stanford University, Stanford, CA, 3Dept. of Structural Biology, Stanford University, Stanford, CA

The development of multicellular organisms requires the orchestrated movement and adhesion of cells that are driven by the regulated assembly and organization of actin networks. Cadherins are key regulators of cell-cell adhesion and are associated with the actin cytoskeleton primarily through α-catenin, which binds the cadherin/β-catenin complex and F-actin. In vitro studies indicated that the oligomeric state of α-E-catenin, the epithelial form of α-catenin, modulates its binding affinities: the monomer forms a ternary complex with β-catenin and E-cadherin, whereas the homodimer preferentially binds to actin filaments and inhibits Arp2/3-mediated actin polymerization. To investigate how α-catenin regulates actin dynamics underlying cell-cell adhesion and cell motility, the cytosolic pool of α-E-catenin was sequestered to mitochondria or the plasma membrane without affecting α-E-catenin levels or the strength of cadherin-mediated cell-cell adhesion. Mitochondrial sequestration of α-E-catenin increased cell migration, lamellipodial dynamics, cortical actin filament polymerization and branched-actin organization. In contrast, recruitment of α-E-catenin to the plasma membrane decreased membrane dynamics, cortical actin polymerization and branched-actin structures. Thus, α-E-catenin functions in cell-cell contact dependent and independent modes to regulate actin dynamics during epithelial cell-cell adhesion and cell migration. In contrast to α-E-catenin, the highly related neuronal form α-N-catenin is predominantly monomeric at concentrations (≈10 μM) that promote α-E-catenin homodimerization. Functional complementation tests conducted in MDCK cells, in which endogenous α-E-catenin expression was silenced using RNAi knockdown, revealed that expression of α-N-catenin rescues cadherin-mediated cell-cell contact formation and cell-cell adhesion defects. Thus, homodimerization does not appear to be required for α-catenin function in cell-cell adhesion. Together, these experiments define new functions for α-catenin and offer further insight into the role of α-catenin in cadherin-mediated adhesion.

13 An Essential Role for p120-Catenin in Vascular Patterning and Endothelial Proliferation.
R. G. Oas1, K. Xiao2, S. Summers1, K. B. Wittich1, C. M. Chiasson1, W. Martin2, H. E. Grossniklaus2, P. A. Vincent7, A. B. Reynolds2, A. P. Kowalczyk1,6; 1Cell Biology, Emory University, Atlanta, GA, 2Transgenic Mouse Core Facility, Emory University, Atlanta, GA, 3Department of Ophthalmology, Emory University, Atlanta, GA, 4The Center for Cardiovascular Sciences, Albany Medical College, Albany, NY, 5Department of Cancer Biology, Vanderbilt University, Nashville, TN, 6Department of Dermatology, Emory University, Atlanta, GA

The armadillo family protein p120-catenin binds to the cytoplasmic domain of classical cadherins and stabilizes them at the cell membrane by preventing their endocytosis. To examine the role of p120 in mouse vascular development, a conditional Cre/loxP gene deletion strategy was used to ablate p120 expression, using the endothelial-specific Tie2 promoter to drive Cre recombinase expression. Mice lacking endothelial p120 died embryonically beginning at E11.5. Major blood vessels appeared normal at E9.5. However, both embryonic and yolk sac vasculature of mutant animals were disorganized and displayed decreased microvascular density by E11.5. Importantly, both VE-cadherin and N-cadherin levels were significantly reduced in vessels lacking p120. This decrease in cadherin expression was accompanied by a reduction in the recruitment of pericytes to developing brain blood vessels and the occurrence of hemorrhages. Primary cultures of p120-null endothelial cells displayed a loss of VE-cadherin expression and revealed proliferation defects that could be rescued by exogenous expression of VE-cadherin. Furthermore, the expression of VE-cadherin, as well as the proliferation defect, could be rescued by the re-
expression of both wild-type p120 and Rho-uncoupled mutant p120. This result suggests that the observed proliferation defect is cadherin-dependent and supports our earlier observation that the internalization of VE-cadherin is Rho-independent. These findings reveal a central and indispensable role for p120 in mammalian vascular development and demonstrate that p120 is required for the regulation of endothelial cadherin levels during vascular development, as well as for microvascular patterning, vessel integrity, and endothelial cell proliferation.

Minisymposium 3: Cell Polarity (14 – 19)

14
Cell Polarity in the C. elegans Embryo.
J. Ahringer; Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, United Kingdom

Cell polarity is critical for many of the functions of animal cells, such as migration, directional secretion, axis formation, and asymmetric cell division. In addition, loss of cell polarity is a contributing factor in cancer. Many of the known molecules involved in generating and transducing cell polarity are conserved across animals, however, the mechanisms by which these function are not well understood and additional cell polarity genes remain to be discovered. The one-celled C. elegans embryo is one of the best-established systems for investigating cell polarity. It allows study of the all of the essential features of cell polarity common in polarized cells: the polarity cue and its reception by the cytoskeleton, the polarisation events that occur in response to the polarity cue, and the transduction of information to downstream polarisation events such as asymmetric spindle positioning. Our long-term goal is to provide an understanding of cell polarity, from delivery of the cue to the observed asymmetries. I will discuss our recent results on cell polarity establishment and transduction in the C. elegans embryo. In addition, I will present our progress on high-throughput RNAi screening for suppressors of a large number cell polarity and asymmetric spindle positioning mutants, with the goal of expanding and making novel connections within the cell polarity network. The experimental amenability of C. elegans will allow new testable models to be interrogated with specific experiments.

15
Microtubules and PAR-2 Break Symmetry to Initiate Polarization of the C. elegans Zygote.
S. A. Zonies, F. Motegi, G. Seydoux; Johns Hopkins University School of Medicine, Baltimore, MD

Microtubules have been implicated in the induction of polarity in several cell types but how they interact with the PAR group of polarity regulators is not well understood. Here we report that microtubules bind the polarity regulator PAR-2, and that this interaction is sufficient to break symmetry and initiate polarization of the C. elegans zygote. Polarization of the C. elegans zygote begins when an asymmetric actomyosin wave sweeps the anterior PAR complex (PKC-3/PAR-3/PAR-6) towards one end of the zygote. While in transit, the PAR proteins amplify the wave (PAR-actomyosin feed-back loop) (Munro et al, 2004). The wave initiates near the sperm-donated centrosome, but the cues that trigger the wave are not known. Using conditions that slow down the wave, we have uncovered a role for microtubules and PAR-2 in this process. PAR-2 is a RING finger domain protein that is excluded from the cortex by direct phosphorylation by PKC-3. We have found that PAR-2 binds microtubules in vitro and in vivo (KDapp<0.2 microM). Microtubule-binding protects PAR-2 from phosphorylation by PKC-3 and allows PAR-2 to localize to the cortex in a small region nearest the sperm centrosome. Once on the cortex, PAR-2 is sufficient to trigger the actomyosin wave. Our genetic analyses suggest that PAR-2 does this by interfering with the PAR-actomyosin feed-back loop. The anterior PAR complex stimulates myosin accumulation on the cortex and PAR-2 antagonizes this activity locally, creating an asymmetric actomyosin contraction. The contraction depletes PKC-3, which in turns allows more PAR-2 to accumulate on the cortex. Our findings provide a simple model for how microtubules can break symmetry by helping PAR-2 overcome cortical exclusion by PKC-3.
Cell polarity is critical for differentiation. A dramatic example of polarization can be found in syncytial cysts of developing *Drosophila melanogaster* spermatids, in which haploid nuclei are found at one end and sperm tail growth occurs at the other. Remarkably little is known about how spermatid polarity is established. Here we identify the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) as a critical regulator of spermatid cyst polarity. Severe reduction of plasma membrane PIP2 in developing male germ cells results in unpolarized spermatid cysts, whereas moderate reduction results in formation of bipolar cysts, with nuclei localizing to both ends during elongation. Cell polarity markers that localize to the growing end in wild-type cysts relocalize to the middle of the cysts when PIP2 levels are reduced. Defects in polarity are evident from the earliest stages of elongation, indicating that PIP2 is required for establishment of polarity. The PIP2 biosynthetic enzyme Skittles (Sktl) and PIP2 colocalize at the growing end of the spermatid cysts with the exocyst complex, which drives targeted membrane addition and cell polarization in a variety of systems. Flies mutant for two exocyst components phenocopy PIP2-depleted flies by showing unpolarized spermatid cysts. Strikingly, the exocyst becomes completely delocalized when PIP2 levels are reduced. Overexpression of Sktl restores exocyst localization and spermatid cyst polarity. Our data are consistent with a mechanism in which localized synthesis of PIP2 recruits the exocyst to promote targeted membrane delivery and polarization of the elongating cysts.

Early embryos of many animals polarize radially when contacts differentiate the outer (contact-free) and inner (contacted) surface of each cell. In *C. elegans*, contacts polarize the embryo radially by spatially regulating Rho GTPase signaling, which induces an inner-outter cortical asymmetry in PAR polarity proteins. In a screen for additional regulators of radial polarity, we identified the *tat-5* gene. *tat-5*, which encodes an aminophospholipid translocase predicted to move phosphatidylserine from the outer leaflet of the membrane bilayer to the inner leaflet, is required for the formation of inner-outter PAR asymmetry. TAT-5 localizes to the plasma membrane and endomembranes of early embryonic cells, and is also needed for proper organization of the plasma membrane at contact sites following cytokinesis as well as for endocytosis of midbodies. These findings suggest a link between membrane organization/composition and the generation of cell polarity in response to cell-cell contact.

Formation of epithelial lumens requires the spatiotemporal co-ordination of apico-basal polarization in cells contributing to the lumen. We here demonstrate that a Rab11a-directed
network co-ordinates apical membrane delivery and single lumen formation in three-dimensional MDCK cyst cultures. Rab11a functions in lumen formation in part by recruiting the Rab GEF Rabin8, co-opting Rab8a to function in apical trafficking. Rab11a also controls Cdc42 and Par3 apical targeting, the latter of which depends on coupling of Rab11a to the exocyst. The exocyst and Par3/aPKC complex assembles at a transient apical fusion patch, allowing surface delivery of apical membrane from Rab11 vesicles. Loss of function of these components disrupts formation of the luminal surface. We thus describe a membrane transport module regulating lumen morphogenesis of multicellular epithelial structures. Moreover, we reveal a novel interplay of the machineries of vesicular transport and apical polarity.

19
Reversal of Neuronal Polarity After Axon Removal: Converting a Dendrite into a Regenerating Axon by Rebuilding the Microtubule Cytoskeleton.
M. C. Stone, M. M. Nguyen, J. Tao, M. M. Rolls; Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA

Axon regeneration is crucial for recovery after trauma to the nervous system. In order for neurons to recover from complete axon removal they must re-specify a dendrite into an axon- a complete reversal of polarity. We find that mature dendrites can reverse polarity and become regenerating axons, and that this involves complete remodeling of the microtubules throughout the neuron. Dendritic microtubules not only reverse polarity, changing from minus ends distal to the cell body (minus-end-out) to plus-end-out, but the number of dynamic microtubules is also dramatically and unexpectedly increased. Both JNK signaling and new microtubule nucleation are required to initiate the response. To assay neuronal responses to axon removal, we use an in vivo Drosophila system and sever axons or dendrites with a UV laser in whole animals. Axon, but not dendrite, injury induces two types of global microtubule rearrangements: 1. 10-fold upregulation of the number of growing microtubules and 2. polarity reversal such that one dendrite takes on the axonal microtubule rearrangement. After microtubule reversal, the process initiates tip growth, and takes on morphological and molecular characteristics of an axon. Growing microtubule number and microtubule polarity were determined by monitoring the number and direction of movement of EB1-GFP comets in dendritic arborization neurons. Reduction of JNK signaling or msp (XMAP215)-stimulated microtubule dynamics blocked repolarization of a dendrite to a growing axon. Using RNAi, we show that downstream of JNK, nucleation of new microtubules is required to initiate the global microtubule rearrangements that lead to re-specification of a mature dendrite to a regenerating axon. Based on our data we conclude that, in vivo, axon injury initiates a specific response that is different from dendrite injury. This response includes a dramatic upregulation of microtubule dynamics, which leads to a complete remodeling of the microtubule cytoskeleton.

Minisymposium 4: Chromatin Organization and Dynamics (20 – 25)

20
Dosage Compensation in Drosophila.
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In Drosophila, dosage compensation of the single male X chromosome involves upregulation of expression of X linked genes in Drosophila. Dosage compensation complex (DCC) or the male specific lethal (ML) complex is intimately involved in this regulation. The MSL complex members decorate the male X chromosome by binding on hundreds of sites along the X chromosome. Recent genome wide analysis has brought new light into X chromosomal regulation. It is becoming increasingly clear that although the X chromosome achieves male specific regulation via the MSL complex members, a number of general factors also impinge on this regulation. Our progress will be presented.
21

O-GlcNAc is Part of the Histone Code.
K. Sakabe, Z. Wang, G. W. Hart; Biological Chemistry, Johns Hopkins University, School of Medicine, Baltimore, MD

The modification of Ser and Thr residues of nucleocytoplasmic proteins by N-acetylglucosamine (O-GlcNAc) is a dynamic post-translational modification. This post-translational modification is absolutely required for life in plants and mammals. More than one thousand GlcNAcylated proteins have been identified ranging from components of the transcriptional machinery including RNA polymerase II, signaling molecules such as kinases and adaptor proteins, ribosomal proteins, and cytoskeletal proteins. Modification with O-GlcNAc is known to regulate protein functions such as protein-protein interactions, activity, and degradation, indicative of its vital role in signal transduction pathways. Here, we report that histones, the basic component around which DNA is wound, are GlcNAcylated. GlcNAc moieties on histones were detected either immunologically or by in vitro labeling with galactosyl transferase. Additionally, histones are substrates for O-GlcNAc Transferase (OGT) in vitro. Previous studies have shown that Coactivator Associated Arginine Methyltransferase 1 (CARM1) targets OGT to specific substrates. Pre-incubating histones with CARM1 increased GlcNAcylation of histones and this increase was not dependent on CARM1 activity. Additionally, knocking-down CARM1 levels in cells using siRNA, reduces the GlcNAcylation of histones, supporting that CARM1 serves to target OGT to histones in vivo. We also identified several O-GlcNAc sites on histones using a chemo-enzymatic approach that tags O-GlcNAc with biotin, enabling facile enrichment of GlcNAcylated peptides. Coupling this technique with Beta-Elimination and Michael Addition of DTT (BEMAD) and LC-MS/MS, we were able to identify several sites on histone H2A, H2B, and H4. The sites identified on H2B and H4 are known phosphorylation sites, while H2A is a novel site found within the core. These findings add O-GlcNAc to the repertoire of post-translational modifications comprising the histone code. Supported by NIH grants DK61671 and CA42486. Dr. Hart receives a share of royalty received by the university on sales of the CTD110.6 antibody. Terms of this arrangement are managed by JHU.

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Double Strand DNA Breaks Recruit Centromere Protein A.
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The histone H3 variant Centromere Protein A (CENP-A) is required to specify centromere location through a loading mechanism independent of DNA sequence. Using multiphoton laser damage and DNA cleavage at unique sites by I-SceI endonuclease, we demonstrate that CENP-A is rapidly recruited to double-strand breaks, along with CENP-N, CENP-T, and CENP-U. The centromere-targeting domain (CATD) of CENP-A is both necessary and sufficient for recruitment to double-strand breaks. CENP-A accumulation at DNA breaks is enhanced by active non-homologous end-joining but does not require DNA-PKcs or Ligase IV, and is independent of H2AX. Thus, induction of a double-strand break is sufficient to recruit CENP-A in human and mouse cells. Finally, since cell survival after radiation-induced DNA damage correlates with CENP-A expression level, we propose that CENP-A may have a function in DNA repair.

23

The MRG15 Chromodomain Protein Physically Interacts with Condensins to Modulate Global Chromosome Architecture and Local Gene Expression.
G. Bosco, H. F. Smith, T. A. Hartl, M. Roberts; Molecular & Cellular Bio, University of Arizona, Tucson, AZ
The dynamic structure and 3-dimensional organization of chromosomes play critical roles in the regulation of gene expression. However, the factors that control global changes in chromosome architecture, and how these factors might impinge on local chromatin structures are not well understood. We have previously shown that Drosophila condensins function to antagonize global homologous chromosome alignment and homolog pairing. Condensins also inhibit the transfer of transcriptional states from one allele to another, such as in the case of transvection at the Ubx and yellow genes. Here we show that the Cap-H2 condensin protein physically interacts with the MRG15 chromodomain protein. Condensins require the MRG15 chromodomain protein in order to effect this chromosome anti-pairing activity. By using 3-D fluorescent in situ hybridizations we find that MRG15 mutants enhance condensin mutant homolog and chromatin pairing phenotypes. MRG15 mutants also enhance transvection at the Ubx gene demonstrating that interchromosomal interactions regulating enhancer-promoter interactions in interphase nuclei are sensitive to MRG15 and condensin activities. Furthermore, we show that the Set2 histone methyltransferase responsible for methylating histone H3 Lysine-36 is also required to recruit MRG15 and condensins to chromatin, and mutations in Set2 or RNAi knock-down of Set2 in Drosophila tissues enhance condensin mutant chromosome structure phenotypes. We propose a model whereby condensins physically interact with MRG15 to regulate global chromosome structure, and Set2 methylation of H3 Lysine 36 marks specific genomic regions to which the MRG15/condensin complex is tethered. This is the first demonstration of any specific chromatin binding factors that are necessary for recruitment and activation of condensin activity. This model also explains how global chromosome maintenance machines, such as condensins, can also function at local and specific gene sequences to modulate gene expression.

24
RNA:DNA Hybrids Control Pericentric Heterochromatin Formation and Chromosome Segregation through the SUV39H2 and SETDB1 Histone Methyltransferases.
L. Strickland, A. F. Straight; Biochemistry, Stanford University, Stanford, CA

Epigenetic regulation of chromatin through histone and DNA modification influences nuclear organization and gene expression but how these modifications are targeted to specific chromosomal sites is poorly understood. We have investigated the role of chromosome associated RNA in the organization and post-translational modification of mitotic chromatin. We isolated mitotic chromosomes from cultured human cells, digested the chromatin with RNases of varying specificity and characterized changes in post-translational histone modifications after RNase digestion. Digestion of RNA:DNA hybrids with RNase H caused the re-distribution of pericentric histone H3 tri-methyl lysine-9 (3me-H3K9) to sites throughout chromosome arms and the release of an H3 histone methyltransferase (HMTase) activity from chromosomes. To identify this activity, we used RNAi to knockdown each of the known human HMTases and screened for the loss of H3-specific HMTase activity after digestion with RNase H. Depletion of SUV39H2 and SETDB1 resulted in loss of the HMTase activity released from chromatin by RNase H digestion, suggesting that association of SUV39H2 and SETDB1 with centromeres is dependent upon an RNA:DNA hybrid. We further found that SUV39H2 and SETDB1 interact with each other and form a complex with HP1 and alpha-satellite RNA. Depletion of SUV39H2 or SETDB1 resulted in chromosome cohesion defects and segregation errors during mitosis. SUV39H2/SETDB1 depletion did not result in activation of the mitotic checkpoint, though RNAi-transfected cells were capable of activating the checkpoint in response to nocodazole or taxol. BubR1 but not MAD2 was recruited to metaphase kinetochores in RNAi-transfected cells. One possibility is that loss of cohesion after SUV39H2/SETDB1 depletion prevented the establishment of tension across bioriented kinetochores, leading to BubR1 recruitment. However, if kinetochores were able to establish microtubule attachments to the mitotic spindle, MAD2 recruitment and full activation of the mitotic checkpoint would not be expected, consistent with our observations.

25
Cis and Trans Determinants of Large-Scale Chromatin Structure.
A. Belmont\textsuperscript{1}, Y. Hu\textsuperscript{1}, W. Wu\textsuperscript{1}, M. Plutz\textsuperscript{1}, Q. Bian\textsuperscript{1}, I. Kireev\textsuperscript{1,2}; \textsuperscript{1}Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, \textsuperscript{2}Electron Microscopy, Moscow State University, Moscow, Russia

How chromatin folds into mitotic and interphase chromosomes remains unknown. Over a number of years we have used engineered chromosome regions tagged with lac operator repeats as a means of visualizing the structure of specific chromosome regions while also facilitating a reductionist experimental approach to this problem. We have progressed from gene amplified chromosome regions, to regions created by multi-copy integration of plasmids, and now to engineered chromosome regions formed by multi-copy BAC repeats carrying 100-200 kb of defined genomic loci. Recent results with the multi-copy BAC constructs suggest that transcription at levels approaching within several fold the level of the corresponding endogenous gene locus occurs on a condensed template which, however, undergoes a global, long-range several fold decondensation with transcriptional induction. Moreover, sequences contained within specific BACs are sufficient to target these sequences to specific nuclear compartments. We have begun to apply these engineered chromosome regions to determine cis and trans determinants of large-scale chromatin structure. Using a Drosophila S2 cell line containing multiple copies of a MT BAC transgene we have used an RNAi screen of over 200 genes related to chromatin structure and transcription to determine the contribution of their gene products on the large-scale chromatin structure of the MT transgene array before and after gene induction. We identify a large number of genes whose knockdown results in increased condensation or increased decondensation before and/or after gene induction. To investigate cis determinants of large-scale chromatin structure, we have begun by dissecting what sequences on a Hsp70 BAC transgene are required to confer nuclear speckle association after heat shock. We show that targeting to nuclear speckles of the Hsp70 BAC transgene is conferred by specific, several kb sequences contained within the Hsp70 BAC. We conclude that engineered chromosome regions can be used to enable specialized screens to investigate specific questions related to large-scale chromatin structure and nuclear organization.

Minisymposium 5: Clocks (26 – 31)

26

The Essence of Time: Biological Clock Nanomachines, DNA Topology, and Fitness.
C. Johnson; Department of Biological Sciences, Vanderbilt University, Nashville, TN

Cyanobacteria have become a major model system for analyzing clock phenomena. The temporal program in this organism enhances fitness in rhythmic environments and is truly global—essentially all genes are regulated by the circadian system. The topology of the chromosome also oscillates and possibly regulates the rhythm of gene expression. The underlying circadian mechanism appears to consist of both a post-translational oscillator (PTO) and a transcriptional/translational feedback loop (TTFL). The PTO can be reconstituted in vitro with three purified proteins (KaiA, KaiB, and KaiC) and ATP. These three proteins rhythmically form a complex that acts as a nanoclock. The three core oscillator proteins have been crystallized and structurally determined, the only full-length circadian proteins to be so characterized. The timing of cell division is gated by a circadian checkpoint, but the circadian pacemaker is not influenced by the status of the cell division cycle. This imperturbability may be due to the presence of the PTO that persists under conditions in which metabolism is repressed. Recent biochemical, biophysical, and structural discoveries bring the cyanobacterial circadian system to the brink of explaining heretofore unexplainable biochemical characteristics of a circadian oscillator: the long time constant, precision, and temperature compensation.

27

A Cyanobacterial Model for How Cells Tell Time.
S. S. Golden; Molecular Biology, University of California- San Diego, La Jolla, CA
Cyanobacteria, like diverse eukaryotes, possess circadian clocks that allow cells to coordinate physiological processes with the predictable daily patterns of environmental fluctuations on Earth. An autonomous oscillator comprised of three distinctive proteins, KaiA, KaiB, and KaiC, underpins the circadian clock of the cyanobacterial model organism *Synechococcus elongatus* PCC 7942. Our work applies genetic, genomic, biochemical, and structural approaches, integrated with cell biology, to achieve a comprehensive understanding of how the *S. elongatus* cell harnesses this oscillator to control cellular activities. The emerging picture is one of a clock that is set daily by sampling the cellular redox environment as a proxy for light and which uses protein-protein interactions and subcellular localization to coordinate gene expression, cell division, and chromosome dynamics. A functional genomics project has produced inactivation alleles of nearly all *S. elongatus* loci. Gene expression assays of the 700 mutants tested so far suggest that about 10% of loci affect circadian period or phasing.

28
**Role of a Jumonji-Domain Containing Protein in Circadian Clock.**
*S. Panda; The Salk Institute for Biological Studies, La Jolla, CA*

Circadian rhythm in behavior and physiology in mammals is generated by a cell autonomous transcription-translation feedback loop. Two bHLH domain containing transcription factors, BMAL1 and CLOCK, heterodimerize and bind to cis-acting E-box element present in the promoter regions of *Period (Per)* 1, 2, and *Cryptochrome (Cry)* 1, and 2 genes and drive their transcription. The PER and CRY proteins in turn repress CLOCK/BMAL1 activity and generate ~24 h rhythms in mRNA and protein levels of PER and CRY gene products. The roles of chromatin modification and chromatin modifying proteins in this transcriptional rhythm are not clearly understood. We have found that a jumonji domain containing protein functions as a co-activator of CLOCK/Bmal mediated transcription. This jumonji protein physically interacts with Clock/Bmal1 in vivo and is recruited to the promoter of Per genes. siRNA knockdown or genetic ablation of this jumonji protein results in significant alteration in chromatin modification in Per promoter leading to reduced amplitude and shortened period length of Per transcription. The results highlight the roles of an emerging class of chromatin modifying proteins in circadian function.

29
**Cellular Basis of Rhythmic Behaviors.**
*A. Sehgal, K. Xu, J. DiAngelo; Department of Neuroscience, University of Pennsylvania School of Medicine/HHMI, Philadelphia, PA*

Circadian rhythms are 24 hour cycles of behavior and physiology found in virtually all organisms. While the best known example of such a rhythm is the sleep:wake cycle, these rhythms pervade most aspects of physiology and are found in clocks located in many peripheral tissues in addition to the brain. Using the fruit fly, *Drosophila melanogaster*, as a model, we showed recently that metabolic functions are controlled by clocks in neurons as well as in the fly fat body (equivalent of the liver and adipose tissue). These two tissues have opposing effects on the storage of nutrient reserves and thereby on food consumption and the response to starvation. A circadian rhythm of feeding is also likely controlled by metabolic and neuronal clocks. To determine how the fat body regulates feeding and metabolism, we profiled gene expression in this tissue at different times of day through microarray analysis. We found cyclic expression of genes that function in several different pathways- lipid metabolism, immune response, detoxification etc. While the cycling of some genes is controlled by the fat body clock, others cycle under the control of clocks elsewhere. Interestingly, metabolic cues can regulate the phase of circadian gene expression in the fat body. Current research is directed towards determining whether synchrony of clocks in different tissues is important for fitness. Key words- Circadian rhythms, genetics, clocks, *Drosophila*, metabolism, feeding

30
**AMP Kinase Regulates the Circadian Clock by Cryptochrome Phosphorylation and Degradation.**
K. Lamia, U. Sachdeva, L. DiTacchio, S. Panda, R. Shaw, C. Thompson, R. Evans; Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, CA; Molecular and Cellular Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA; Regulatory Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA; Abramson Family Cancer Research Institute and Department of Cancer Biology, University of Pennsylvania School of Medicine, Philadelphia, PA; Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, CA; Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, PA

Circadian clocks coordinate behavioral and physiological processes with daily light-dark cycles by driving rhythmic transcription of thousands of genes in mammalian tissues. While the master clock in the suprachiasmatic nucleus is set by light, pacemakers in peripheral organs are reset by food availability. In either case, the setting mechanism remains mysterious. Light-dependent clock resetting in plants and insects depends on blue light-driven destabilization of cryptochromes. In mammalian clocks, cryptochrome degradation is mediated by the F-box protein FBXL3 in a light-independent manner. We demonstrate that the nutrient-responsive AMP-activated protein kinase (AMPK) phosphorylates the clock component CRY1 and destabilizes it by increasing its interaction with FBXL3. AMPK activity and nuclear localization are rhythmic, and inversely correlated with CRY1 protein abundance. Stimulation of AMPK destabilizes cryptochromes and alters circadian rhythms. Genetic inactivation of AMPK blocks these effects and disrupts peripheral clocks. Thus, AMPK-dependent phosphorylation enables light-resistant cryptochromes to act as chemical energy sensors and likely contributes to metabolic entrainment of mammalian circadian clocks.

31
Src-Family Tyrosine Kinase Phosphorylation Directs the Degradation of the Clock Protein Timeless via Ubiquitylation and c-Cbl Interaction.
L. O'Reilly, T. E. Smithgall; Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA

The circadian clock, an endogenous internal timekeeping system, imposes a 24 hour cycle regulating the essential functions of metabolism and behavior. The molecular basis of the clock involves an autoregulatory loop composed of cycling gene products that control their own synthesis. Drosophila have the ability to reset their circadian clock by light-dependent degradation of the central clock protein, Timeless. Timeless is proteosomally degraded via association with Cryptochrome, which recruits the E3 ubiquitin ligase Jetlag, thus relieving inhibition of transcription and resetting the circadian clock. Timeless degradation is preceded by tyrosine phosphorylation, although the identity of the kinase responsible for this key event triggering the degradation pathway has remained elusive. In mammals, the circadian clock is more complex. Although mammalian Timeless associates with the circadian protein Period and exhibits 24-hour oscillation, Timeless has not yet been definitively placed in the mammalian circadian clock. However mammalian Timeless appears to have essential functions in cell cycle control and embryonic development. Here we report a novel interaction of mammalian Timeless with members of the Src-family of non-receptor protein-tyrosine kinases. Timeless interacts with the SH3 domains of the Src-family members Hck, Fyn, c-Yes and c-Src. Co-expression with each of these kinases in 293T cells led to tyrosine phosphorylation of Timeless. Phosphorylation, particularly with c-Src, enhanced Timeless ubiquitylation. In contrast, treatment of 293T cells with Src-family kinase inhibitors blocked Timeless degradation. As protein-tyrosine phosphorylation is known to recruit the E3 ubiquitin ligase c-Cbl, we also investigated whether c-Src-mediated phosphorylation would recruit c-Cbl to Timeless as part of the proteosomal degradation pathway. Co-expression of Timeless, c-Src and c-Cbl did indeed enhance Timeless degradation, which was blocked with the proteosomal inhibitor ubiquitin aldehyde. Our data demonstrate that SFK-mediated tyrosine phosphorylation directs the proteosomal degradation of Timeless in a light-independent manner, which may be important for Timeless turnover related to non-circadian functions.
Minisymposium 6: Intracellular Trafficking (32 – 37)

32
A Novel Sec24 Mutant Links Cargo Capture to the GTP Cycle of the COPII Coat.
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Vesicle budding from the endoplasmic reticulum is initiated by the G-protein Sar1p, which is converted from a soluble GDP-bound form to a membrane-associated GTP-bound form. Sar1p:GTP recruits the cargo adaptor complex, Sec23p/Sec24p, which in turn recruits the outer coat complex, Sec13p/Sec31p. Following vesicle release, the coat must be shed to permit vesicle fusion. Coat disassembly is an intrinsic property of the COPII coat, in that the GTP cycle on Sar1p is regulated by components of both the inner (Sec23p) and outer (Sec31p) layers of the coat. The GTPase activity of the full COPII coat creates a paradox whereby rapid coat disassembly in the presence of GTP may preclude the creation of a coat complex of sufficient stability to recruit cargo proteins and deform the membrane. In studying the cargo-binding capacity of the Sec24p subunit, we have discovered a novel mutation that confers a severe secretion block and renders cells temperature-sensitive when present as the sole copy of Sec24p. This mutant protein, Sec24p-A11, is perfectly capable of budding vesicles in vitro in the presence of GMP-PNP, but is dramatically impaired in its ability to support vesicle formation in the presence of GTP. This phenotype is reminiscent of vesicle formation in the absence of Sec16p, which similarly cannot support GTP-dependent budding. Cells expressing sec24-A11 are synthetic lethal with sed4Δ and sec16-2 mutants, and the Sec24p-Sec16p interaction detected by yeast 2-hybrid analysis is perturbed by the A11 mutation. We propose that association of Sec24p with Sec16p retards the GTPase activity of the Sar1p/Sec23p/Sec31p complex such that when this interaction is perturbed (by A11 mutation or removal of Sec16p from membranes), GTP hydrolysis occurs prematurely and productive vesicle budding is reduced. Indeed, in vitro GTPase assays demonstrate that a fragment of Sec16p has GAP inhibitory activity that is abrogated by the A11 mutation. We propose that Sec24p represents a critical regulatory factor that couples cargo selection (via the known cargo-binding sites) to GTP hydrolysis (via Sec16p interaction at the A11 site).

33
Transporting Cholesterol out of the Lysosome: A Pocket-to-Pocket Handoff from NPC2 to NPC1.
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Mammalian cells acquire cholesterol (chol) by receptor-mediated endocytosis of LDL. Once within lysosomes, LDL’s cholesterol esters are hydrolyzed by acid lipase. The released chol is transported across the lysosomal membrane to the ER and plasma membrane where it performs regulatory and structural functions. Exit of chol from lysosomes requires two proteins: NPC1, a 1278-amino acid (aa) polytopic membrane protein; and NPC2, a 131-aa soluble protein. Mutations in the genes encoding either of these proteins produce an excessive accumulation of chol in lysosomes, leading to a fatal neurodegenerative disease called Niemann-Pick C. Purified NPC2 and the soluble 240-aa N-terminal domain (NTD) of NPC1 both bind chol with high affinity, yet their binding kinetics differ: NPC2 binds chol rapidly, whereas NPC1(NTD) binds slowly. NPC2 accelerates binding of chol to NPC1(NTD) by >100 fold. X-ray crystallography of NPC1(NTD), together with alanine scan mutagenesis, reveal two distinct subdomains: one for chol binding (buried pocket) and the other for chol transfer (exposed helices). NPC1(NTD) binds chol with its 3β-hydroxyl buried and isooctyl side chain exposed. This orientation is opposite to the binding of chol within the NPC2 pocket. Entry of chol into the binding pocket of NPC1(NTD) is hindered by a narrow constriction point, requiring its opening by reorientation of helices within the
transfer subdomain. Point mutations in this subdomain prevent pocket-to-pocket chol transfer from NPC2 to NPC1(NTD). When NPC1-deficient fibroblasts were transfected with cDNAs encoding full-length NPC1 with point mutations in either the binding or transfer subdomain, chol exit from lysosomes was not restored. We propose the following model: after lysosomal hydrolysis of LDL’s cholesteryl esters, chol binds NPC2 (isooctyl side chain buried; 3β-hydroxyl exposed), opening the constriction point in NPC1(NTD) and permitting chol transfer to NPC1(NTD) (3β-hydroxyl buried; isooctyl side chain exposed). This transfer reverses chol’s orientation, allowing insertion of its isooctyl side chain into lysosomal membranes - possibly through the interaction of the NTD of NPC1 with its own membrane domain.

34 Building the Primary Cilium Membrane: Regulation of GEF Trafficking and Activity and a Rab11-Rab8 Cascade.

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Aberrant primary cilia function/formation have been linked to human genetic diseases, including Bardet-Biedl syndrome (BBS). A core complex of BBS genes, the BBSome, has been proposed to function in vesicle trafficking during cilium formation, by binding to Rabin8, a Rab8 guanine nucleotide exchange factor (GEF), and which in turn activates Rab8 to promote ciliogenesis. We study here how membrane trafficking events regulate Rab8-dependent ciliary membrane biogenesis. Using a stably expressed GFP-Rab8 fusion, we find Rab8 rapidly relocating to the basal body in RPE cells following initiation of ciliation induced by serum withdrawal. Rab8 assembles into a pericentriolar preciliary vesicle, which extends and elongates during cilia growth for ~100 minutes, and is then lost from mature cilia. Remarkably, Rab8-dependent preciliary/ciliary membrane formation is preceded by microtubule-dependent vesicular transport of Rabin8 to the centrosome, observable within 10 minutes of serum depletion. Using purification/mass spectrometry and a yeast two-hybrid screen with a library of GTP-locked Rabs, we discover that Rabin8 preciliary trafficking is controlled by association with Rab11-GTP and the TRAPP II complex, endosome recycling compartment (ERC) regulators. Rab11 and the TRAPP II complex bind to distinctive domains on Rabin8. We also demonstrate that another ERC regulator EHD1, a membrane bending protein, is required for ciliation. Interestingly, EHD1 localizes distinctively to the proximal region of the ciliary membrane, and MEFs from EHD1 knockout mice have shorter and fewer cilia. Investigation into BBSome involvement in ciliary trafficking showed that BBS1 modulates Rab8 activation by binding to the Rabin8 GEF domain. BBS1 depletion stimulates Rab8 activation resulting in the formation of Rab8 membrane tubules and also increases the length of mature cilia. A Rab11-Rab8 cascade model is proposed, wherein Rabin8 is trafficked by Rab11-positive vesicles to the centrosome, leading to BBS-regulated activation of Rab8, which targets preciliary vesicles and EHD1 to generate the polarized ciliary membrane.

35 Interplay Between Epsins and Sla2 Is Essential for Endocytosis in Yeast.

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Clasthrin-mediated endocytosis is driven by a complex and highly dynamic molecular machinery. The ENTH/ANTH domain proteins epsins and Sla2 (homolog of mammalian HIP1/HIP1R) are essential for actin-dependent formation of endocytic vesicles. We used live-cell fluorescence microscopy and genetics to elucidate the endocytic roles of epsins Ent1/2 and Sla2 in Saccharomyces cerevisiae. We first used FRAP (fluorescence recovery after photobleaching) to
systematically assess the exchange rates of endocytic proteins at endocytic sites when endocytosis was arrested by latrunculin A (latA). The recovery half-times varied from ~3 s to >2 min. The most stable proteins were the Pan1 complex (Pan1/Sla1/End3) and ENTH/ANTH proteins Ent1/2, Sla2 and Yap1801/2. Notably, all these stable factors are associated with the endocytic coat. To confirm our results with a different method of endocytic arrest, we repeated the FRAP analysis in sla2 null cells, where the actin polymerization at endocytic sites is uncoupled from membrane invagination. While the turnover of most proteins did not change in comparison to the latA experiment, the turnover rates of epsins increased dramatically, suggesting that Sla2 stabilizes epsins at the endocytic site. In addition, Ent1 colocalized with endocytic actin structures revealing an unexpected interaction of epsin with the actin cytoskeleton. We then analyzed cells where both redundant epsins were depleted. The epsin depletion resulted in an endocytic phenotype identical to sla2 deletion. Expression of epsin fragments in epsin depleted cells showed that both the ENTH domain and the C-terminal part of the protein localized to endocytic sites, but only the ENTH domain was able to rescue the endocytic function. Notably, the localization of the ENTH domain, but not the C-terminal part, was lost in the absence of Sla2 protein. These results strongly suggest that Sla2 is required for stable association of epsin ENTH domains with the endocytic membrane. The Sla2-dependent stabilization of epsin ENTH domain in the endocytic coat, together with the direct link of Ent1 and Sla2 to the actin cytoskeleton, could be fundamental for transmitting force from the actin meshwork to bend the membrane.

A Novel, Evolutionarily Conserved Rab5 Effector Controls Rab GTPase Conversion on the Surface on Phagosomes.

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Engulfment of apoptotic cells occurs throughout life in multi-cellular organisms as part of normal development, homeostasis, and wound healing. Impaired clearance of apoptotic cells due to defects in recognition, internalization or corpse degradation results in autoimmune diseases (such as lupus and chronic polyarthritis) linked to exposure of auto-antigens. Understanding how defects in corpse removal translate into diseased states critically depends on identifying players that orchestrate different steps of engulfment. Here, through a combination of genetic, biochemical and cell biological studies in worm, yeast and mammalian cells, we identify SAND−1 (and its orthologue MON1) as a critical evolutionarily conserved player during phagosome maturation. In sand−1 deficient worms, apoptotic cells are properly internalized, but phagosomes containing apoptotic cells are arrested at the RAB−5(+), PtdIns3P(+) stage, prior to RAB−7 recruitment. Similarly, mammalian MON1a and MON1b are jointly required for acidification of apoptotic cell containing phagosomes. Mechanistically, we define the requirement for SAND−1 (MON1) in at least three steps of phagosome maturation. First, MON1 specifically interacts with Rab5(GTP), identifying MON1 as a novel Rab5 effector. Second, we find that MON1, in complex with another protein CCZ1, can interact with Rab7; thus, by associating with active Rab5(GTP), CCZ1 and Rab7, MON1 provides a protein:protein interaction bridge from active Rab5(GTP) to Rab7, suggesting a mechanism for Rab7 recruitment. Lastly, we show biochemically and in cells that MON1:CCZ1 facilitates nucleotide release from Rab7. Taken together, we identify SAND−1 (MON1) as a distinct type of Rab5 effector and suggest a novel molecular mechanism for the conversion of Rab5 to Rab7 on the surface of the phagosome.

Regulation of Golgi Division and Mitotic Progression by Golgi Matrix Proteins.

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The mammalian Golgi apparatus is comprised of a series of stacked cisternae that are interconnected into a ribbon-like network in the pericentriolar region. Two protein families of the Golgi matrix, GRASPs and Golgins, play essential roles in maintaining the integrity of the Golgi ribbon as well as tethering the vesicles during intra-Golgi transport. In mitosis the Golgi complex
is disassembled and partitioned into the nascent daughter cells where a functional Golgi reassembles. Upon mitotic entry, matrix proteins are phosphorylated by mitotic kinases, which facilitates the rapid disassembly of the Golgi. Moreover, these proteins also regulate spindle assembly and mitotic progression. The disassembled mitotic Golgi membranes accumulate around the spindle poles, implying a role for the spindle in Golgi partitioning. By inducing asymmetrical cell division where the spindle is segregated into only one of the daughter cells, we show that the inheritance of the higher-ordered Golgi ribbon depends on the mitotic spindle. Taken together, we present the mutual influence between the Golgi apparatus and the spindle machinery during mitosis.

Minisymposium 7: Regulation of Cell Growth (38 – 43)

38

**Growth Control by the mTOR Pathway.**

*D. Sabatini; Whitehead Institute for Biomedical Research/MIT/HHMI, Cambridge, MA*

mTOR is the target of the immunosuppressive drug rapamycin and the central component of a nutrient- and hormone-sensitive signaling pathway that regulates cell growth and proliferation. We now appreciate that this pathway becomes deregulated in many human cancers. We have identified two distinct mTOR-containing protein complexes, one of which regulates growth through S6K and another that regulates cell survival through Akt. These complexes, mTORC1 and mTORC2, define both rapamycin-sensitive and insensitive branches of the mTOR pathway. I will discuss new results from our lab on the regulation of the mTORC1 and mTORC2 pathways. I will provide an overview of mTOR signaling as well as discuss the regulation of mTORC1 by insulin and nutrients and the role of mTORC2 in cancer. We have recently identified new upstream components of the mTORC1 pathway that are involved in amino acid sensing and are starting to understand some of the molecular mechanisms involved in this process. Current evidence suggests that amino acids regulate the mTORC1 pathway by controlling the intracellular localization of mTORC1. Specifically, amino acids promote the movement of mTORC1 to a part of the endomembrane system that also contains its activator Rheb. Amino acids signal through the conserved Rag family of small GTPases that directly interact with the raptor component of mTORC1 in fashion that depends on the GTP-loaded status of the Rags. In addition, I will present evidence that inhibition of mTORC1 by the novel mTOR-interacting protein Deptor is a mechanism for hyperactivating PI3K signaling in multiple myeloma.

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**hVps34 Activates PLD1 Upstream of mTORC1.**

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The mammalian target of rapamycin (mTOR), in the distinct rapamycin-sensitive protein complex mTORC1, is a critical regulator of cell growth in response to mitogen and amino acids signals. Phospholipase D (PLD) and its product phosphatidic acid (PA) have been established as upstream regulators of mTORC1 mediating mitogenic signals. Another upstream regulator is the class III phosphatidylinositol 3-kinase hVps34, which has been reported to contribute to the regulation of mTORC1 by amino acids. Our recent finding that PLD1 is regulated by amino acids, combined with the fact that PLD1 harbors a PX domain—a potential target of the hVps34 enzymatic product PI3P, prompted us to examine a relationship between hVps34 and PLD1. Here, we find that hVps34 is required for amino acids activation of PLD1, as demonstrated by the effects of RNAi. Exogenous PA rescues amino acids- and insulin-induced mTORC1 activation from the negative effects of hVps34 knockdown, suggesting that PA (and PLD) lies downstream...
of hVps34 in the regulation of mTORC1. Furthermore, overexpression of hVps34 activates PLD1 in a dose dependent manner, and this activation requires the PX domain in PLD1, as a PX-deleted PLD1 mutant does not respond to hVps34. Consistent with the involvement of PI3P, overexpression of a FYVE domain impairs hVps34-induced PLD activation. We also find that Vps34 physically interacts with PLD1, and that this interaction is independent of mitogens or amino acids. Taken together, these observations strongly suggest that PLD1 mediates hVps34 activation of mTORC1 in response to amino acids. Our findings reveal a novel molecular interaction in the amino acid-sensing mTOR signaling pathway and identify a new regulator for PLD1.

40  
Mechanisms Linking Cell Geometry and Mitotic Entry in Fission Yeast.  
J. Moseley\(^1\), A. Mayeux\(^2\), A. Paoletti\(^2\), P. Nurse\(^1\); \(^1\)The Rockefeller University, New York, NY, \(^2\)Institut Curie, Paris, France

Many eukaryotic cell types undergo size-dependent cell cycle transitions controlled by the ubiquitous cyclin-dependent kinase Cdk1. The proteins that regulate Cdk1 activity are well described but their links with mechanisms monitoring cell size, shape, and geometry remain elusive. Here we describe a protein network that controls mitotic entry through Cdk1 and receives signaling inputs related to cell geometry in the fission yeast S. pombe. The core of this network is located in the middle of interphase cells at cortical nodes that contain the Cdk1 inhibitor Wee1 and the Wee1-inhibitory kinases Cdr1/Nim1 and Cdr2. Cdr2 establishes the hierarchical localization of other proteins in the nodes, and receives negative regulatory signals from Pom1, a conserved cell polarity kinase. Pom1 forms a polar gradient extending from the cell ends towards the cell middle and acts as a dose-dependent inhibitor of mitotic entry, working through the Cdr2 network. As cells elongate, Pom1 levels decrease at the cell middle to promote mitotic entry. Because pom1\(^{Δ}\) cells do not enter mitosis as prematurely as wee1 mutant cells, we performed genome-wide screens for additional inputs to the Cdr2-Wee1 network. This identified novel regulators of Cdr2 that display additive genetic defects with pom1\(^{Δ}\) and localize to distinct cortical structures. We propose that combined signals from these structures and the Pom1 polar gradient generate a measurement of cell geometry that controls mitotic entry through Cdr2, Cdr1, and Wee1. Such a system may prevent errors in chromosome segregation, as cell geometry determines the orientation and behavior of the mitotic spindle in a range of cell types including fission yeast.

41  
Control of Organ Size by the Hippo Signaling Pathway.  
D. Pan; Department of Molecular Biology & Genetics, Johns Hopkins University School of Medicine/HHMI, Baltimore, MD

How tissues reach and maintain their characteristic size is a long-standing puzzle in developmental biology. In recent years, we have defined a novel kinase cascade, the Hippo pathway, which plays a critical role in stopping organ growth as cells enter the differentiation phase of organogenesis. In Drosophila, the core of the Hippo kinase cascade comprises the Ste20-like kinase Hippo (Hpo), the NDR family kinase Warts (Wts), and the transcriptional coactivator Yorkie (Yki). Hpo phosphorylates and activates Wts, which in turn, inactivates Yki by phosphorylating the latter and excluding it from the nucleus, where it normally functions as a coactivator for the TEAD/TEF family transcription factor Scalloped (Sd). The Hippo pathway promotes cell death and restricts cell proliferation through the transcriptional regulation of target genes such as the cell cycle regulator cyclin E and the cell death inhibitor diap1. Very recently, we have delineated a mammalian Hippo pathway that links the mammalian homologues of Hpo (MST1/2), Wts (Lats1/2) and Yki (YAP) in an analogous kinase cascade. As in Drosophila, we found that the mammalian Hippo pathway inactivates YAP by excluding it from the nucleus. Using a conditional transgenic mouse model, we showed that YAP induction results in a robust and uniform expansion of liver size to five times normal, followed by rapid progression to hepatocellular carcinoma. These observations implicate the Hippo pathway as a universal size-
control mechanism in flies and mammals, and further revealed a causal link between dysregulation of this size-control pathway and tumorigenesis. We have conducted a cell based RNAi screen to identify additional components of the Hippo pathway in Drosophila. I will present genetic characterization of a novel component of the Hippo pathway.

42
Identifying Targets of the Hippo Tumor Suppressor Pathway that Regulate Growth and Proliferation.
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The hippo tumor suppressor pathway regulates growth, proliferation and apoptosis and is highly conserved from Drosophila to humans. The upstream kinase Hippo phosphorylates other components of the pathway including the downstream kinase Warts. Warts phosphorylates downstream targets such as yorkie (YAP in mammals) and DIAP1, but few other targets have been identified. We performed a genome-wide kinase substrate screen to identify additional downstream targets of the Hippo and Warts kinases. From more than 11,000 gene products screened in small pools, we identified hippo itself as proof of principle. We repeated and identified 36 individual positive hits from roughly 100 positive pools. These in vitro substrates fall into different categories including regulators of endocytosis, mitosis, and protein biosynthesis. We pursued substrates with a putative role in mitotic progression while we continue to identify substrates from the remaining positive pools. Here we report that hps3 (Hippo Pathway Substrate 3) is phosphorylated in vitro and degraded upon hippo over-expression in S2 cells, suggesting its phosphorylation promotes its degradation as seen with DIAP1. Loss of one copy of hps3 in vivo enhances the growth restriction phenotype of hippo over-expression in the eye. Reduction of hps3 using RNAi results in smaller organ size in both the eye and the wing. The reduced eye size is suppressed by mutation in hippo but not by caspase inhibition, suggesting that down-regulation of hps3 mediates the hippo pathway’s role in growth and/or proliferation, but not apoptosis. Our preliminary phenotypic characterization and genetic interaction studies suggest that hps3 regulates organ growth and may inhibit important mitotic exit components. Our model is that (1) hps3 phosphorylation by Warts promotes its instability and that (2) regulation of growth and proliferation by the hippo pathway is in part achieved by down-regulating hps3 to ensure proper organ size control and to upregulate mitotic exit activities. Like yorkie, other targets of the hippo pathway may play an important role in regulating growth and proliferation and could represent novel tumor suppressors and oncogenes.

43
A Novel Mechanism for Cell Size Control by Regulators of the Retinoblastoma Tumor Suppressor Pathway.
J. Umen, B. Olson, C. Lopez, Y. Li; Plant Biology Laboratory, The Salk Institute, La Jolla, CA

Proliferating cells maintain size homeostasis through mechanisms that are thought to involve size checkpoints, but the nature of such checkpoints has been difficult to determine. The unicellular alga Chlamydomonas reinhardtii is a uniquely accessible model for cell size control owing to its multiple fission cell cycle where growth and division are uncoupled. The retinoblastoma (RB) tumor suppressor pathway is a key mediator of Chlamydomonas size checkpoints, but upstream components of the RB pathway that control its activity in response to cell size are unknown. We isolated and characterized two new size checkpoint mutants, cdkg1 (large phenotype) and tny1 (small phenotype), that were both upstream of RB (encoded by MAT3) in a linear genetic pathway. CDKG1 was found to encode a novel algal-specific cyclin dependent kinase (CDK) whose mRNA abundance was cell cycle regulated. CDKG1 over-expression led to a small-cell phenotype indicating that CDKG1 is a rate-limiting size regulator. Interestingly, CDKG1 interacted with Chlamydomonas D-type cyclins in vitro, and recombinant CDKG1-cyclin D complexes could phosphorylate MAT3/RB. Our findings indicate a remarkable evolutionary convergence between
CDKG1 and the independently evolved metazoan kinases CDK4/6 and CDK2 as regulators of the RB pathway. TNY1 was found to be genetically upstream of CDKG1 and encodes a cytoplasmic hnRNP-related RNA binding protein that acts as a cell cycle repressor. Using highly synchronous cultures we determined that TNY1 protein is made once per cell cycle at the time of division, and then remains at a constant absolute level during G1. This pattern of synthesis suggests a model for size checkpoint control where a constant amount of the repressor TNY1 is diluted by an increasing amount of its target RNAs, one of which is likely to be encoded by CDKG1 whose unusually long and uridine-rich 3’ untranslated region is a putative TNY1 target. This titration model involving tightly regulated production of repressors such as TNY1 that are diluted by cell growth to allow synthesis of activators such as CDKG1 may be a widespread principle for coupling cell growth to cell cycle activation or other critical processes.

Bruce Alberts Award (44 – 45)

44

Advancing Science Literacy among Under Represented Minorities.
M. P. Berriozábal; Department of Mathematics, University of Texas at San Antonio, San Antonio, TX

In 1979 Berriozábal received a grant from the U. S. Department of Energy to start the San Antonio Prefreshman Engineering Program (PREP) at the University of Texas at San Antonio. The seven-to-eight summer mathematics-based enrichment program for mostly underrepresented minority middle and high school students focuses on developing their abstract reasoning and problem-solving skills. Ultimately, it prepares and encourages many of the students to pursue college-level work in science, technology, engineering and mathematics (STEM). The program expanded in 1986 to other colleges throughout Texas; it expanded again in 1997 to other states with a continuing focus on minority students, particularly Hispanics. The program has received numerous awards and is considered one of the top K-12 STEM programs in the U.S. To date over 28,000 students are former PREP participants. Results show that 99% of students who attend a PREP summer session graduate from high school; of those, 99% go on to college, 82% graduate from college, and 46% of the college graduates are STEM majors.

45

A Creative, Rewarding Career Pathway for Scientists: Teaching Pre-College Science.
T. M. Horn; Carnegie Academy for Science Education, Carnegie Institution for Science, Washington, DC

In this century of science and technology, pre-college programs around the country include S&T, but so many students in our urban and rural localities experience science only as "reading the textbook and writing vocabulary." Localities with specialized schools for science that are populated with high achievers and independent schools hire S&T professionals and support laboratory science learning. This was how, in part, Thomas Jefferson High School for Science and Technology (TJ) developed into the school rated #1 nationally by USNews for two years running. But, our country is filled with talented students from urban and rural centers who, though deserving, have been woefully underprepared. DC schools are surrounded by S&T industries, yet the nation's Capital has about the very lowest ranked school system. At TJ, starting in 1985, we developed a series of short courses (like MBL, CSHL) in a rotation with other technologies that introduced 400 9th grade students annually to basics of biotech including microbiology, plant and animal cell culture, enzymology, and molecular biology. Later courses included plant tissue culture, DNA biotech and Senior Research and Mentorship. The program has evolved to include the interests of new faculty. Seniors intern in labs all over the DC Metro area. At McKinley Technology HS and Ballou Senior High School, in NE and SE Washington DC, our group at CASE is assisting in the development of career pathways in biotechnology. The DC school system has outfitted these labs generously. In 20 years, new technologies like thermal cyclers and digital microscopes are becoming available even at the pre-college level, but students still
need to learn the basics of handling themselves and the tools, materials and equipment. Science connects all the subjects and provides interesting context for reading and math, areas where the US is lagging internationally. We scientists know how to DO science. It feels as good when a student "gets it" as when the data from our experiment show a pattern we can interpret. We can't let the kinds of science learning non-opportunities of so many students AND pre-service teachers continue. Would YOU be satisfied if someone told you most days, "This is the book. You are not allowed to read this book. I haven't read the book either, but I will tell you what's in it. You will take tests answering the information I gave you." We well-educated scientists have much to offer to our youth and colleagues who teach youngsters. Teaching is not an alternative career for a scientist; it's a rewarding pathway.

E. E. Just Lecturer (46)

46
Nicotinic ACh Receptors in the Brain; Structure, Function and Role in Disease.
J. L. Yakel; National Institute of Environmental health Sciences (NIEHS), Research Triangle Park, NC

The human brain contains hundreds of billions of neurons, all interconnected at synapses, the specialized cellular contacts which transmit electrical messages that allow the mind and body to continually sense and respond to the myriad of stimuli in the environment and the other organ systems. Thus, dysfunctions in neuronal signaling, either through disease, injury, or toxic/environmental insult, are a major health hazard. Neurons signal one another at synapses by releasing chemicals neurotransmitters that bind to receptor proteins on the surface of neighboring cells. Neurotransmitter receptors regulate neuronal function by activating ligand-gated ion channels (LGICs; single protein complexes that include both the receptor binding site and the ion channel pore) which rapidly excite or inhibit ongoing electrical activity. In the Ion my group, we study the LGIC superfamily, with a particular emphasis on the nicotinic acetylcholine (ACh) receptor channels (nAChRs). These channels are permeable to cations, and some are also permeable to calcium ions, and this increase in cytoplasmic calcium levels ([Ca^2+]_i) can affect signal transduction cascades, plasticity, and gene transcription. The nAChRs are widely expressed (in neurons and in non-neuronal cells) throughout the brain, both on postsynaptic membranes, where they mediate synaptic transmission by cholinergic neurons, and on presynaptic terminals of non-cholinergic neurons, where they regulate release of other neurotransmitters. The nAChRs are essential for a variety of fundamental brain functions, including cognitive processing, learning and memory formation, and development. Dysfunctions in nAChR function have also been linked to a variety of neurological disorders and diseases, including AD, Parkinson’s disease (PD), epilepsy, and schizophrenia. The goal of my group is to elucidate the role nAChRs play in the pathology of neurological diseases and disorders. Besides understanding the pathology of serious diseases afflicting millions of Americans, this information may also aid in the development and design of therapeutics to treat or mitigate the symptoms of nAChR dysfunction.

Symposium 1: The Human Genetics as Two-Way Information (47 – 49)

47
Mechanosensation in Auditory Sensory Cells: What Did We Learn From Deafness Genes?
C. Petit; ¹Neuroscience, College de France & Institut Pasteur, Paris, Paris Cedex 15, France, ²Inserm UMRS587 - UPMC, Paris 75015, France

Mechanosenses are the most numerous senses but the least understood, mainly because their sensory cells are either very few or scattered throughout tissues. The genetic approach is particularly suitable to resolve this issue. Recent advances in the identification of the genes underlying early onset forms of hearing impairment in humans and the successful application of
multidisciplinary analyses of their mouse models enlightened the way in which the auditory hair bundle develops and functions, and launched the deciphering of the involved molecular mechanisms. The hair bundle, the most sophisticated microvillar structure of the animal kingdom, is a femtoliter cellular compartment of thirty to a few hundred stiff microvilli, improperly called the stereocilia, inter connected by fibrous links and with a shape unique for each auditory sensory cell. It responds with a sharpened frequency selectivity to sound mechanical stimulus, transduces it with an extreme sensitivity and distorts the sound waves that it processes. The remarkable capacities of the hair bundle are associated to extreme vulnerability of this structure. Most of the genes defective in human deafness affect the auditory sensory cells. In most cases, the hair bundle is the primary target of the defect and myosin motors form the prevailing class of the proteins encoded by theses genes. Based on the large group (about 30 genes defects) of hair bundle developmental anomalies observed, a view of the various clues that control the hair bundle shaping is emerging. Currently, the most unexpected results came from the deciphering of the molecular composition of the hair bundle links, the various subsets of lateral links and the tip link, a component of the transduction machinery. The study of Usher syndrome (sensory neural deafness associated to blindness) provided major insights. The proteins encoded by the 9 known genes responsible for Usher type I and II, form molecular complexes that compose two subsets of transient lateral links critical for the hair bundle morphogenesis. Several Usher type I proteins also later make up the core of the mechanoelectrical transduction machinery.

48
V. Sheffield; University of Iowa/HHMI, Iowa City, IA

The identification of genes, sequence variations, and mechanisms involved in complex human disorders holds great promise for improving health care, but at the same time presents a difficult challenge to the scientific community. To better understand the genetics of complex human disorders, my laboratory has studied Mendelian (monogenic) disorders that share a phenotypic component with common complex disorders. We have used human populations to map dozens of disorders and have used positional cloning methods to identify numerous disease-causing genes. Recent work in our laboratory has focused on the study of Bardet-Biedl syndrome (BBS). BBS has the primary features of obesity, retinal degeneration, polydactyly, hypogonadism, renal anomalies, and mental retardation. Secondary features of BBS include diabetes mellitus, hypertension, and congenital heart defects. Studies by us and others have shown that there are more than 12 genes that independently can cause BBS syndrome. Current efforts are aimed at determining the pathophysiology of specific components of the BBS phenotype, including the function and interactions of the known BBS genes, and defining the components and specific interactions of BBS protein complexes. Animal models have implicated abnormal cilia function in the pathogenesis of BBS. Mice lacking Bbs gene expression have major components of the human phenotype, including obesity and hypertension. Our genetic, cell biological, and animal model work is aimed at understanding and intervention in human disease.

49
Genes that Control the Shape and Size of the Human Cerebral Cortex.
C. Walsh; 1Childrens Hospital Boston, Boston, MA, 2Harvard Medical School, Boston, MA, 3Howard Hughes Medical Institute, Boston, MA

The large human population of the world, and the widespread occurrence of mutations that cause neurological disease, make the study of human genetic disorders of cerebral cortical development a rich source of genes essential for neural development. Improved imaging of the human brain has revolutionized our understanding of brain malformations. Between 5-40% of epilepsy patients show malformations of the cerebrum, which are especially common in children with severe epilepsy. It is increasingly common to find subtle malformations of the cortex in people of normal intelligence. Moreover, other disorders affect synaptic function without causing a gross malformation, producing mental retardation, autism, or other learning disorders. Genetic
studies in our lab and others in the last few years have identified many genes that cause human malformations—LIS1, DCX, FLNA, ARFGEF2, Reelin, POMT1, ASPM, CENPJ, CDK5RAP2, GPR56, etc. However, we are just starting to understand the rich variety of disorders of brain development. Identified genes encode proteins with key roles in proliferation of neuronal precursor cells or migration of neurons, two essential features of the development of our cerebral cortex. Many patients with developmental brain disorders have “de novo” mutations, meaning that the patient is the first in the family to manifest the malformation and the mutation, analogous to cancer, in which a “genetic disease” is not usually inherited in a simple way. The greatest phenotypic and genetic diversity of developmental brain disorders represent rare recessive conditions, for which many genetic causes still remain undiscovered. Continued identification of genes that cause human cortical disorders promises to inform us about how our brain develops and even how it evolved, since a larger and more highly ordered cerebral cortex is a principal feature that distinguishes us from other mammals. Surprisingly, some of the genes essential for human cortical development also appear to have been targets of the evolutionary selection that distinguishes our brain from that of other animals.

Symposium 2 - Under the Hood of the Cell: Dynamic Organelles (50 – 52)

50 How Mitochondria Divide and Fuse.
J. Nunnari; Department of Molecular and Cellular Biology, University of California, Davis, Davis, CA

Mitochondria are essential organelles that perform a myriad of tasks within eukaryotic cells. Unlike their bacterial ancestors, they are not discrete entities; rather, isolated organelles are transient and are in constant communication with each other via fusion. Mitochondrial division antagonizes fusion and together these events fundamentally serve to create a compartment that is connected and thus functional, yet able to be distributed via transport on cytoskeletal networks. In mammals, additional roles for mitochondrial fusion and division proteins have evolved by virtue of their integration with cellular signaling networks. The best characterized is cell death, where these proteins directly modulate mitochondrial dependent apoptosis. Mitochondrial division and fusion are mediated by the action of highly conserved dynamin-related proteins (DRPs) that, through their ability to self-assemble and hydrolyze GTP, control membrane remodeling events. We have developed experimental systems to elucidate how DRPs function to mediate mitochondrial division and fusion as well as to uncover the mechanism of how these events are regulated in cells.

51 Epistatic Mini Array Profiles (EMAPs) Reveal that the Asthma-Associated ORM/ORMDL Family Proteins are Key Mediators of Sphingolipid Homeostasis.
J. Weissman; Department of Cellular and Molecular Pharmacology, University of California, San Francisco/HHMI, San Francisco, CA

Despite the essential role of sphingolipids as both structural components of membranes and critical signaling molecules, we have a limited understanding of how cells sense and regulate their levels. Here we reveal the function of the ORM/ORMDL genes, a conserved gene family that includes ORMDL3, a recently identified potential risk factor for childhood asthma. Starting from an unbiased functional genomic approach based on an analysis of large scale genetic interaction networks, we identify Orm proteins as negative regulators of sphingolipid synthesis that form a conserved complex with serine palmitoyltransferase, the first and rate-limiting enzyme in sphingolipid production. We also define a regulatory pathway in which phosphorylation of Orm proteins relieves their inhibitory activity when sphingolipid production is disrupted. Changes in Orm gene expression or mutation to the Orm phosphorylation sites cause dramatic dysregulation of sphingolipid metabolism. Our work identifies the Orm proteins as critical mediators of...
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sphingolipid homeostasis and implicates misregulation of sphingolipids in the development of childhood asthma.

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Autophagosome Biogenesis and Function.
J. Lippincott-Schwartz; National Institute of Child Health and Human Development/NIH, Bethesda, MD

Autophagy is a highly conserved, bulk degradation pathway in eukaryotes that includes turnover of large aggregates and organelles within cells. In this pathway, activation of specific autophagy effectors, including LC3, induces a membrane bilayer to wrap around portions of the cytoplasm to form a double membrane-bounded structure called the autophagosome. The autophagosome then targets to and fuses with the lysosome where the sequestered materials (including protein aggregates or organelles) are degraded by various hydrolytic enzymes and recycled as amino acids for macromolecule synthesis and energy production. Emerging results have revealed the importance of autophagy in various biological and pathological processes, such as cellular remodeling, tumorogenesis and neurodegeneration. However, how this pathway operates and the characteristics of its central organelle (i.e., the autophagosome) are far from understood. We have utilized in cellulo pulse-chase labeling with photoactivatable fluorescent proteins, photobleaching, electron microscopy tomography, and other techniques to investigate whether autophagosomes derive from preformed membrane(s) or by a de novo process, the signals that recruit substrates to autophagosomes, and whether selective types of autophagosomes are used for different cellular functions.

Porter Lecturer (53)

53

A Journey with Molecular Motors.
R. Vale; Department of Cellular and Molecular Pharmacology and HHMI, University of California, San Francisco, San Francisco, CA

Ever since I was a graduate student, I have been “hooked” on understanding biological motion, a problem that also fascinated Keith Porter. In the mid 1980s, I never thought that one could hope to understand biological motility with any satisfying degree of detail. However, unanticipated tools of single molecule measurements, genomic sequencing, and protein expression have allowed our field to probe and understand how molecular motors work with extraordinary precision. I will provide a brief scientific tour of these past twenty years and then focus on a couple of questions on the dynein motor that occupy our present attention. Interwoven with the science, I will highlight some lessons that I have learned along the way. I have had the good fortune of being guided into this profession by a number of wonderful scientists and mentors, and I hope to emphasize the joy and privilege of being part of the scientific discovery process.

Parasitology (54 – 68)

54/B1

FRET Peptides Reveal Differential Proteolytic Activation in Intraerythrocytic Stages of the Malaria Parasites Plasmodium berghei and P. yoelii.
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Malaria is still a major health problem in developing countries. It is caused by the protist parasite Plasmodium in which proteases are activated during the cell cycle. In this context, Ca²⁺ is an
ubiquitous signaling ion that tightly regulates protease activity through changes in its intracellular concentration. Proteases are crucial to Plasmodium development but the role of Ca^{2+} in their activity is not fully understood. In the present work we are interested in determining the role of Ca^{2+} in protease modulation among different Plasmodium species. Using Fluorescence Resonance Energy Transfer Peptides (FRET) we verified protease activity elicited by Ca^{2+} from the endoplasmic reticulum (ER) after stimulus with thapsigargin (SERCA inhibitor). Classes of affected proteases were investigated with the class-restricted protease inhibitors PMSF (serine proteases), E64 (cysteine proteases) and Pepstatin a (aspartic proteases) after thapsigargin treatment. Intracellular Ca^{2+} chelator (BAPTA/AM) was used to adjust Ca^{2+} concentration. In Plasmodium berghei and P. yoelii, Ca^{2+} released from the ER activated proteolysis of FRET peptides. Effects of protease inhibitors on thapsigargin-induced proteolysis differed between species, Pepstatin a and PMSF increasing proteolysis in P. berghei, but Pepstatin a decreasing it in P. yoelii. E64 reduced proteolysis in P. berghei after thapsigargin, but stimulated it in P. yoelii. The absence of intracellular Ca^{2+} greatly increased peptide hydrolysis in P. yoelii. We concluded that in malaria parasites there are Ca^{2+} modulated species specific proteases and in P. berghei and P. yoelii proteases inhibited by Ca^{2+} are major components of peptide hydrolysis.

55/B2
Macrophage Plasma Membrane Hyperpolarization Associates with Protection against Apoptosis of Leishmania Infected Macrophages.
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Leishmania are intracellular parasites that reside within endo-lysosome compartments of macrophages. We have shown that infection with Leishmania amazonensis or Leishmania braziliensis alters the electrical properties of macrophages. Established parasite infection (more than 48 hours post-infection with parasite replication) induces increases in macrophage membrane capacitance, hyperpolarization of the macrophage plasma membrane associated with increases in inward current density. Hyperpolarization has been associated among others with protection against apoptosis in other cell models and there is evidence that Leishmania infection protects macrophages against apoptosis. In this work we study a model of apoptosis in the murine macrophage cell line J774A.1 with cytochrome C as inducer. Apoptosis was assessed by DNA fragmentation and phosphatydilserine externalization. Macrophage electrical properties were recorded using the whole cell configuration of the patch clamp technique in control, pre-apoptotic, apoptotic and Leishmania infected macrophages before and after cytochrome C treatment. We found that apoptosis does not alter macrophage membrane capacitance, therefore the volume changes associated with apoptosis are not accompanied by plasma membrane area reduction. The plasma membrane potential of apoptotic macrophages was 25% more depolarized than control cells. This depolarization was paralleled by an increase of 59.6% in outward current density with no changes in inward currents. Nevertheless, macrophages infected with Leishmania braziliensis were hyperpolarized compared to control and apoptotic macrophages and protected macrophages against apoptosis induced by cytochrome C around 50%.

56/B3
CLC Channels of the Protozoa Leishmania.
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Leishmania spp. alternate during their life cycle in environments with extreme differences in pH. Promastigotes propagate in the alimentary tract of the sandfly vector, which could be alkaline (pH 7.0-9.0) or acidic (pH 5.0-6.0) and amastigotes reside in a highly acidic environment (pH 4.5-5.5) within the host cell, both maintaining an internal pH close to neutral. The expression of chloride channels in these parasites has been shown coupled with proton pump activity. We have shown chloride currents after the injection of Leishmania amazonensis and Leishmania braziliensis mRNA into Xenopus laevis oocytes using voltage clamp. In this study we show for the first time CLC chloride channels of Leishmania, which may be related to the adaptation and survival of the
parasite to the severe environmental conditions found within the host cell. Immunofluorescence images of *L. braziliensis* using an unspecific CLC antibody showed a speckled cytoplasmic pattern consistent with the location of CLC channels in intracellular organelles and in the flagellar pocket. Primers for putative chloride channel genes were designed based on the sequences available for *L. braziliensis* at the GenBank and the GeneDB. Total RNA was extracted from *L. braziliensis* (HOM BR/75M2903) promastigotes with Trizol, cDNA was synthesized with Superscript II and used as template for RT-PCR. The PCR product was purified, cloned into the pGEMT easy vector and sequenced. The sequence showed high homology to the LbrMO1_V2.0210, GeneID: 5412354 from *L. braziliensis* and phylogenetic analysis revealed that it belonged to the eukaryotic branch of intracellular membrane ClCs. Both, the identified putative CLC gene and the immunofluorescence assays confirmed the presence of at least one CLC chloride channel in *L. braziliensis*. Electrophysiological studies of transfected HEK293 cells with a CT-GFP expression vector coding the putative CLC chloride channel using the patch clamp technique showed currents similar to those observed in *X. laevis* oocytes using the voltage clamp technique, being the first description of a CLC chloride channel of *Leishmania* that could be used as a therapeutic target.

**57/B4**

Large Phagocytic Loads Explain Altered Cytokine Secretion and Cytoskeleton Distribution in Macrophages Infected with *Leishmania*.

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*Leishmania* spp, are intracellular parasites that reside within phagosomes of macrophages. Parasites decrease macrophage nitric oxide production. We have shown that large phagocytic loads of latex beads also decrease nitric oxide production of activated macrophages. This decrease did not associate with lower mRNA or protein levels of inducible nitric oxide synthase but with altered intracellular distribution of this enzyme. Decrease secretion of interleukin 12 (IL-12) and Tumoral necrosis factor alpha (TNF-a) have also been described in macrophages infected with *Leishmania*. In this study we tested the hypothesis that large loads associate with decrease secretion. We determine mRNA levels by real time PCR, protein levels by western blot, secretion by ELISA and flow cytometry and intracellular distribution of IL-12, TNF-a, actin and tubulin. Activation of macrophages is required for expression of IL-12 and its location is submembranal. *Leishmania* infection does not induce expression of IL-12 but reduces it and alters its intracellular distribution after macrophage activation. There is constitutive production of TNF-a mRNA by control macrophages, that increases with activation but decreases with large loads (*Leishmania* or latex beads). TNF-a distribution is perinuclear in control and activated cells where it is also towards the periphery of the cell and is altered by large loads (*Leishmania* or latex beads). TNF-a secretion is significantly decreased also by large loads (*Leishmania* or latex beads) in activated macrophages. Actin distribution of control macrophages is towards the periphery of the cell and tubulin is cytoplasmic and perinuclear. Activation induces more actin processes and increases perinuclear tubulin location. The levels of actin and tubulin measured by western blot are not altered by large loads (*Leishmania* or latex beads), but their distribution is. Together these results suggest that large phagosomal loads alter distribution of intracellular compartments within the macrophage and compromise secretion.

**58/B5**

**CYP5122A1, A Novel Cytochrome P450 Is Essential for Survival of Leishmania Donovani Both In Vitroand In Vivo.**

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Cytochrome P450s (CYP450) are a versatile superfamily of hemeproteins that are known to be involved in drug metabolism/resistance as well as in carrying out important physiological functions. Although CYP450s are known to be crucial for survival of higher eukaryotes, their functional contribution to the physiology of the kinetoplastid parasites are not known. This study
presents evidence for the crucial role of a novel CYP50, the CYP5122A1 in the survival and infective abilities of Leishmania donovani, the parasite that causes visceral leishmaniasis for which there are distinct endemic pockets throughout the world and the disease is fatal if left untreated. The Leishmania donovani CYP5122A1 (DQ267494), localized to the endoplasmic reticulum of the parasite, shows 87-94% similarity to the orthologues in different members of genus Leishmania while having only 17-19% similarity with human CYP450s. Complete knockouts of CYP5122A1 did not survive while half knockouts (HKOs) with one allele of the gene intact and the other replaced with the neomycin gene survived, although they grew more slowly than the wild-types. These parasites with half the amount of protein as compared to the wild type cells showed higher susceptibility to the anti-leishmanial drug miltefosine. Since miltefosine induced higher expression of CYP5122A1 in wild-type cells, the compromised ability of the HKOs to survive miltefosine exposure together suggested that CYP5122A1 could be related to miltefosine action. The HKOs were also susceptible to oxidative stress, a common agent generated during drug action and also during parasite invasion of the macrophage. Understandably, the HKOs were unable to sustain infection in macrophages as efficiently as the wild-type cells, both in vitro in macrophage cell line J774 and also in vivo in macrophages from hamster infection model. Since CYP450s are also known to be involved in sterol biosynthesis, ergosterol which is the primary sterol in Leishmania membrane was estimated and the HKOs showed the presence of lesser ergosterol (~50%) than the wild-types. Taken together, the data suggest that CYP5122A1 is an essential component for the survival of Leishmania donovani.

59/B6
Purine Salvage by the Human Pathogen Leishmania mexicana Is Facilitated by a Unique Secretory Nuclease; LmexNUC<sup>s</sup>
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All Leishmania spp reside and multiply as extracellular promastigotes (Pro) within the midgut of their sandfly vectors and as intracellular amastigotes (Am) within the phago-lysosomal system of human macrophages. These parasites are obligate purine auxotrophs and, in order to survive and multiply, they must salvage such essential molecules from their hosts. Here, we identified and characterized a unique 35kDa secretory nuclease (LmexNUC<sup>s</sup>) which might play a significant role in purine acquisition by <i>L. mexicana</i>. Sequence analysis revealed that LmexNUC<sup>s</sup> possesses a signal peptide and five structural motifs characteristic of the P1/S1 fungal/plant secretory nuclease family. Northern blot and protein analyses confirmed that LmexNUC<sup>s</sup> was transcribed and differentially translated through the parasite’s life cycle (Ax>>Pro). Western blot and enzyme activity analyses verified that LmexNUC<sup>s</sup> was constitutively secreted/released by both <i>L. mex</i> Pro and Am developmental forms. In order to delineate the functional properties of the LmexNUC<sup>s</sup>, the gene was episomally over-expressed in <i>Lmex</i>NUC<sup>s</sup>-HA transfectants. Results of combined anti-HA immunoprecipitation/enzyme activity assays showed that LmexNUC<sup>s</sup> was N-linked glycosylated and that it could readily degrade RNA, ssDNA as well as various synthetic polynucleotide substrates (i.e. poly-A, -I, -U). Further we demonstrated that LmexNUC<sup>s</sup> was irreversibly inactivated by sulphhydral reducing agents e.g. DTT. Cumulatively, our results indicate that LmexNUC<sup>s</sup> must play important roles in facilitating both the growth and development of this important human pathogen.

60/B7
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Immune cell factors of the severe chronic chagasic human heart disease were reported; previous work showed occurrence of CD4-T-lymphocyte population, high producers of P.A.S.-positive glycoproteins, with evidences suggesting a role in damages in myocardium and neural structures.
in chagasic heart (Cabral, Lancet 1:1356, 1971; Cabral, Novak, Mem Inst Oswal Cruz, 90 S I:157, 1995, 92, S I, 1997). Interferon Gamma production by such cells of chagasic patients was reported recently (Cabral et al, 2006, Rev Fac Cienc Med Cordoba, 63: 47-52). Occurrence of thick ring nuclei in neutrophils of chagasic patients was reported (Cabral, Br J Haematol, 67: 118, 1987), and the cytological diade: thick ring nuclei in neutrophils and lymphocytes with P.A.S.-positive substance were described in vivo (Cabral et al, Mem Inst Oswal Cruz 83 S I: 95, 1990). Ring-shaped nuclei in human neutrophilic leukocytes in blood of healthy individuals and their wide occurrence in age groups were showed (Cabral et al, Mol Biol Cell. 7 Suppl ASCB, 656a, 1996).

Objective: in the present work we studied cell cultures of total leukocytes to assess such cytological diade. Blood was obtained from 6 patients with positive serological tests for Chagas and 10 healthy controls. Total leukocyte cultures were prepared in TC 199 medium. Samples were undertaken at 0, 5, 8 hours. Likewise for all groups blood fresh smears were prepared and occurrence of ring nuclei neutrophils was assessed. H/E and cytochemical reactions (mieloperoxidase, P.A.S.) were made. With respect to ring nuclei neutrophils, the results showed a significative increase in smears from chagasic patients as compared with controls (p<0.01). Mieloperoxidase showed positive reactivity in ring nuclei neutrophils in all groups and in cultures. The production of lymphocytic P.A.S. positive substances was showed in cultures of all patients. Conclusions: the facts suggest that the above mentioned leukocytic activities in Chagas may be more intense and they occurred in vitro, also.

61/B8
Condensation-Dependent Sorting of a Single-Type Cargo in Differentiating Giardia: A Minimal Model for Cyst Wall Formation.
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The trophozoite stage of the protozoan parasite Giardia lamblia has a basic but effective trafficking system that lacks a Golgi apparatus. for transmission to new hosts, trophozoites differentiate to cysts by secreting a simple extracellular matrix composed of the three members of the cyst wall protein (CWP) family and GalNAc homopolymer chains. CWPs are sorted away from constitutively secreted proteins during ER export and accumulate in organelles called encystation-specific vesicles (ESV). ESVs are the only Golgi cisterna analogs in Giardia and are generated de novo in a COPII-dependent process. This unique system which transports only cyst wall material in differentiating cells corresponds to an inducible and pulsed version of a Golgi according to the cisternal maturation model. Based on this we predicted an as yet unknown sorting step before regulated secretion of ESVs contents. Here, we show that proteolytic processing of CWP2 leads to partitioning of this protein into two fractions with distinct physical states. Using FRAP analysis we demonstrate formation of a condensed core driving cargo partitioning in ESVs and sorting of this material into two different vesicle types by a "sorting by retention" mechanism. In line with genomic evidence for dramatic reduction processes in Giardia during evolution, this constitutes a minimal system for cargo sorting resulting in sequential secretion and polymerization of this material to form a two-layered extracellular matrix. Regulated export of CWPs in differentiating Giardia is the simplest model to investigate basic mechanisms for Golgi neogenesis, protein sorting, as well as ordered assembly of protective biopolymers.

62/B9
Transcriptomics of the Giardia lamblia Degradome.
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Proteolytic activity is important in the life cycle of parasites and in the interactions with their hosts. While the main class of proteases studied in the protozoan parasite Giardia lamblia has been the
cysteine type, the activity of other protease classes have been implicated in the cell cycle and differentiation. To define the complete set of Giardia proteases (degradome), we queried the Giardia genome database for peptidase homologues and we profiled their transcription expression in the different stages of the Giardia life cycle by serial analysis of gene expression (SAGE). We uncovered 87 candidate peptidase genes that were distributed over 5 catalytic classes: cysteine (43.68%), metallo (21.8%), serine (16.1%), threonine (16.1%), aspartic (1.15%), and unknown type (1.15%). Out of the 87 protease ORFs, 11 did not have SAGE tags and 14 had significant transcriptional variation. Only one protease transcript in the serine catalytic family was upregulated in encystation specifically. This serine protease was most similar to eukaryotic subtilisin-like proprotein convertases (SPC). However, this giardial SPC (gSPC) was annotated as a 'non-peptidase homologue' by MEROPS because a classic catalytic triad was not evident. To analyze gSPC protein expression in encystation, we expressed gSPC under its own promoter in a vector with an AUI epitope tag. Western blot analyses showed that the epitope-tagged gSPC protein was upregulated and processed during encystation with highest expression in cysts. Gelatin zymography showed catalytic activity during encystation. Overall our data show that 1.74% of the annotated Giardia genome is composed of peptidases (1.18%) and their homologues (0.56%), which is in range of any cellular genome. Despite the lack of a conserved catalytic domain, the gSPC homologue is catalytically active supporting the inclusion of 'non-peptidase' homologues in our degradome data set. This study is the most complete quantitative analysis of protease gene expression in Giardia to date. These data give direction for future research of specific protease families and their functions.

63/B10
Protein Tyrosine Phosphorylation in the Human Pathogen Giardia lamblia.
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The protozoan parasite Giardia lamblia is a common cause of diarrheal disease worldwide. Infection is initiated by ingestion of dormant cysts. After passage through the stomach, cysts quickly differentiate into motile trophozoites. Trophozoites colonize the small intestinal mucosa and can differentiate into cysts (encystation) that survive outside the human body. Differentiation is tightly regulated and is associated with major morphological changes. Protein phosphorylation and dephosphorylation regulate important biological processes including differentiation. Previous studies have shown that serine/threonine phosphorylation plays and important role in Giardia differentiation. In order to evaluate tyrosine phosphorylation in Giardia, we affinity purified tyrosine phosphorylated proteins from total lysates and immunolocalized and studied their protein expression levels in the life cycle along with the single protein tyrosine phosphatase (PTP). Proteomic analysis identified a number of tyrosine phosphorylated peptides belonging to proteins with a variety of cellular functions. Immunofluorescence analysis demonstrated that in vegetative trophozoites most tyrosine phosphorylated proteins are localized in the nuclei while PTP is present in the basal bodies and posterior-lateral paraflagellar rods. Immunofluorescence and Western blot analysis demonstrate that tyrosine phosphorylation is reduced in encystation and PTP is reduced in cysts. Our results indicate that despite the absence of specific tyrosine kinases in the Giardia genome (Morrisson et al., 2007), a substantial number of Giardia proteins are tyrosine phosphorylated by dual specificity kinases. In contrast to the serine/threonine protein phosphatase 2A (Lauwaet et al., 2007), the localization of PTP did not change during encystation. Future studies are needed to study the role of tyrosine phosphorylation in the Giardia life cycle.

64/B11
Nf-Kappab P65-Dependent Transactivation of miRNA Genes Following Cryptosporidium Parvum Infection Stimulates Epithelial Cell Immune Responses.
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Cryptosporidium parvum is a protozoan parasite that infects the gastrointestinal epithelium and causes diarrheal disease worldwide. TLR/NF-kappaB-mediated epithelial immune responses are key mediators of the host's defense to C. parvum. MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level and are involved in regulation of both innate and adaptive immune responses. Here we tested the role of miRNAs in the regulation of TLR/NF-kappaB-associated epithelial anti-C. parvum defense. Using an In Vitro model of human cryptosporidiosis, we analyzed C. parvum-induced miRNA expression in biliary epithelial cells (i.e., cholangiocytes). Our results demonstrated differential alterations in the mature miRNA expression profile in cholangiocytes following C. parvum infection. Database analysis of C. parvum-upregulated miRNAs revealed potential NF-kappaB binding sites in the promoter elements of a subset of miRNA genes. We demonstrated that miR-125b-1, miR-21, miR-30b, and miR-23b-27b-24-1 cluster genes were transactivated through promoter binding of the NF-kappaB p65 subunit following C. parvum infection. In contrast, C. parvum transactivated miR-30c and miR-16 genes in cholangiocytes in a p65-independent manner. Importantly, functional inhibition of selected p65-dependent miRNAs in cholangiocytes increased C. parvum burden. Thus, we have identified a panel of miRNAs regulated through promoter binding of the NF-kappaB p65 subunit in human cholangiocytes in response to C. parvum infection, a process that may be relevant to the regulation of epithelial anti-C. parvum defense.

**65/B12**

MicroRNA-98 and Let-7 Modulate Cryptosporidium Parvum-Induced Expression of Suppressor of Cytokine Signaling-4 (SOCS4) in Biliary Epithelial Cells.

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Expression of the cytokine-inducible Src homology 2 protein (CIS) and suppressors of cytokine signaling (SOCS) proteins represents an important element of epithelial reactions in response to pathogen infection. Cryptosporidium parvum is a protozoan parasite that infects both intestinal and biliary epithelial cells. MicroRNAs (miRNAs) are small RNA molecules of ~23 nucleotides that negatively regulate protein-coding gene expression via translational suppression or mRNA degradation. Our previous studies demonstrated that C. parvum infection downregulates miR-98 and let-7 to induce CIS expression in human biliary epithelial cells (cholangiocytes)(J Immunol. 2009;183:1617). In this study, we demonstrated that C. parvum infection induced SOCS4 expression in cholangiocytes using an In Vitro model of human biliary cryptosporidiosis. We first identified complementarity of miR-98 and let-7 to the 3'-untranslated region of SOCS4 mRNA. Targeting of SOCS4 3'-untranslated region by miR-98 or let-7 resulted in translational repression, but not mRNA degradation. Functional manipulation of miR-98 caused reciprocal alterations in cellular SOCS4 protein expression. Importantly, transfection of miR-98 precursor abolished C. parvum-stimulated SOCS4 protein expression. In addition, expression of SOCS4 in cholangiocytes caused an inhibitory feedback effect on the phosphorylation of STAT6 induced by C. parvum. Our results indicate that C. parvum infection induces SOCS4 expression in cholangiocytes by downregulation of miRNA-98 and let-7, suggesting an important role of miRNAs in the regulation of epithelial response to C. parvum infection.

**66/B13**

Disruption of a Mitochondrial Muts DNA Repair Enzyme Homolog Confers Drug Resistance in the Parasite, Toxoplasma gondii.

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MutS homologs (MSHs) are critical components of the eukaryotic mismatch repair machinery. In addition to repairing mismatched DNA, mismatch repair enzymes are known in higher eukaryotes to directly signal cell cycle arrest and apoptosis in response to various DNA damaging agents. Accordingly, mammalian cells lacking certain MSHs are resistant to chemotherapeutic drugs. Interestingly, we have discovered that the disruption of TgMSH-1, an MSH in the pathogenic parasite, Toxoplasma gondii, confers drug resistance. Through a genetic selection for T. gondii...
mutants resistant to the anticoccidial drug monensin, we have isolated a strain that is resistant not only to monensin but also to salinomycin and the alkylating agent, methylnitrosourea. We have shown that this phenotype is due to the disruption of \textit{TgMSH-1} as the multi-drug resistance phenotype is complemented by a wild-type copy of \textit{TgMSH-1} and is recapitulated by a directed disruption of this gene in a wild-type strain. We have also shown that, unlike previously described MSHs involved in signaling, \textit{TgMSH-1} localizes to the parasite mitochondrion. These results provide the first example of a mitochondrial MutS Homolog that is involved in drug sensitivity and implicate the induction of mitochondrial stress as a mode of action of monensin. Interestingly, the parasite-specific electron transport inhibitor, decoquinate prevents monensin-induced death in wild-type parasites, further implicating the parasite mitochondrion as the target for monensin. Preliminary analysis of microarray studies has shown that wild-type parasites exposed to monensin have increased levels of several histone mRNAs, which are known to be up-regulated during S-phase of the cell cycle. We are currently investigating monensin’s potential arresting effect on the parasite cell cycle and the role \textit{TgMSH-1} plays in signaling such an arrest.

67/B14

**Role of Calreticulin in \textit{Entamoeba histolytica} Phagocytosis.**

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\textit{Entamoeba histolytica} is an enteric amoeba that is an important cause of dysentery in developing countries. Phagocytosis of host cells is characteristic of invasive amoebiasis. Identification of receptors that aid in phagocytosis is important from the standpoint that they may be new vaccine candidates. In this regard, mass spectrometry analysis of phagosomal proteins led to the identification of several proteins including the endoplasmic reticulum lumen chaperone, calreticulin. Based on studies in macrophages, where surface calreticulin binds to the collagenous tail of C1q and collectins present on apoptotic cells and bacteria, we proposed that a similar mechanism may enable amoebic trophozoites to phagocytose host cells during invasion of the intestinal wall. Previous results that support our hypothesis include an increase in phagocytosis by amoebae overexpressing calreticulin and an increase in staining of epitope tagged calreticulin on the surface of amoebic trophozoites following stimulation with human lymphocytes. Using an antibody directed against native calreticulin, the protein was shown to be recruited to the phagocytic cup following stimulation with erythrocytes. In non-permeabilized cells, calreticulin at the phagocytic cup colocalizes with a galactose specific lectin, which is a membrane marker. These results are consistent with directed recruitment of calreticulin from the ER to the amoeba:host cell interface during phagocytosis. Studies are underway to silence calreticulin by RNAi and to confirm the above results using biochemical approaches. Calreticulin being an ER luminal protein, we are also interested in determining the mechanism by which it reaches the phagosomal cup.

68/B15

**A 330 kD Viral Structural Protein Is a Herpesvirus Microtubule Motor Receptor.**

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Pseudorabies virus (PRV) is a member of the neuroinvasive group of alpha-herpesviruses, which also includes such important pathogens as herpes simplex virus (HSV) type 1 and 2, as well as varicella-zoster virus (VZV). Microtubule (MT)-based transport of these viruses is not only important for the success of the replication cycle and the establishment of latency, but also for the symptomatic development of herpesvirus infection ranging from sores and shingles to more severe forms, such as herpes keratitis and encephalitis. All these processes involve both dynein and kinesin motors, and finding a virus receptor for these motors is very important for both better understanding the biology of the virus and for development of future therapeutic targets. Here we show that a conserved structural protein, VP1/2, binds both kinesin and dynein motor complexes. Interaction with conventional kinesin depended upon a central region of the the VP1/2 protein. Interestingly, this central region of VP1/2 was not sufficient for the kinesin I interaction, which was also dependent on a carboxy terminal region of VP1/2 that we show recruits the dynactin
complex. Moreover, we show that the C-terminus of VP1/2 co-pellets with polymerized tubulin and decorates MTs in transiently transfected cells. VP1/2 is a capsid binding protein, and we therefore propose that VP1/2 connects the capsid to MTs and MT-associated motors to mediate intracellular and axonal transport of the viral particle.

**Cancer I (69 – 102)**

**69/B16 ABSTRACT WITHDRAWN**

**70/B17**

**Caveolin-1 Is an Efficient Tumor Suppressor In Vivo in the Absence of E-Cadherin.**

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Objective: Caveolin-1 has frequently been attributed a role as a tumor suppressor. Data from this laboratory showed that caveolin-1 in the presence of E-cadherin suppresses β-catenin/Tcf-Lef-dependent transcription of survivin and cyclo-oxygenase 2 (COX2), two genes associated with tumor cell survival. However, whether E-cadherin is required for caveolin-1-mediated tumor suppression In Vivo is unknown. Methodology: B16-F0 cells, a melanoma cell line isolated from C57BL/6 mice that express readily detectable levels of caveolin-1 and metastastic B16F10 cells that express extremely low endogenous caveolin-1 levels were employed in this study. Both cell types lack endogenous E-cadherin. Caveolin-1-expressing derivatives of the B16F10 cell were generated by stable transfection. Alternatively, endogenous caveolin-1 expression in B16F0 cells was reduced using specific short hairpin RNA (shRNA) constructs. Tumor volume was recorded in syngenic C57BL/6 mice injected subcutaneously with 300,000 cells. Results: Animals inoculated with B16-F10 cells develop tumors within 21 days. In B16F10 cells expressing caveolin-1, tumor formation was substantially reduced. Alternatively, the reduced tumor forming ability of B16F0 cells in comparison to B16F10 cells was enhanced in B16F0 (shcav-1) cells, coincident with reduced presence of endogenous caveolin-1. Conclusions: The presence of caveolin-1 in the absence of E-cadherin, in either B16F0 or B16-F10 melanomas, reduced the tumor forming ability of these cells in C57BL/6 mice. Acknowledgements: FONDECYT 1090071, FONDAP15010006 (AFGQ), FONDECYT 1070699 (LL) and CONICYT (LL-G, LA).

**71/B18**

**Soluble E-Cadherin Acts as an Anti-Apoptotic Protein Signaling via Epidermal Growth Factor Receptor.**

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E-cadherin is a calcium-dependent cell-cell adhesion molecule in epithelial cells and alterations in E-cadherin function have been linked to carcinogenesis in a variety of cancers. Recent studies revealed that E-cadherin not only functions in cell-cell adhesion but also interacts and modulates several signal transduction pathways. In addition to its well-known interaction with β-catenin, a component of the Wnt signaling pathway, there is considerable evidence that E-cadherin functionally interacts with epidermal growth factor receptor (EGFR) at the sites of cell-cell contact. The exact role of this association is unclear. Clinical studies identified a soluble form of E-cadherin in sera of patients with different types of carcinoma. Soluble E-cadherin results from cleavage of the E-cadherin extracellular domain by matrix metalloproteinases, releasing it from the cell membrane into the extracellular milieu. Despite the wealth of clinical data available, little is known whether soluble E-cadherin has any patho-physiological significance or contributes to cancer progression. In this study we provide evidence that a clinically relevant dose of a recombinant form of soluble E-cadherin (SECAD) prevents cell death due to serum withdrawal.
and functions as a potent inhibitor of apoptosis. Moreover, at concentrations that had no effect on cell-cell adhesion, SECAD prevented normal development of polarized acini, a process that involves programmed cell death. We further show that SECAD mediates its anti-apoptotic effect through activation of EGFR and its respective downstream pathways. Thus, these findings demonstrate a novel role for SECAD in cell survival and suggest that soluble E-cadherin present in patients' sera might contribute to cancer progression.

72/B19

Reduced Tumorigenicity of Cervical Cancer Cells through the Regulation of Axl Signaling Pathway.
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Axl receptor tyrosine kinase is originally known as homeostatic regulators for antigen presentation cells as well as regulator for cell survival, proliferation, and migration, but little is known of the regulatory role in the tumorigenicity of cervical cancer. To elucidate the role of Axl on the human papillomavirus (HPV) E6-mediated tumorigenicity in cervical cancer, HPV type 16 E6-overexpressing HeLa cells (HE6F) and suppressing Caski cells (CE6R) were established. Axl expression was markedly up-regulated in HE6F cells compared to HeLa cells. The overexpressed HPV 16 E6 gene in HE6F cells increased S phase of the cell cycle. Furthermore, apoptosis of HE6F cells were decreased by triggering of Axl signaling pathway. Consistently, stimulation with agonistic anti-Axl increased in the metastasis of Caski cells as well as the cell proliferation in a dose-dependent manner. These findings suggest that Axl plays a critical role in the regulation of HPV 16 E6-mediated tumorigenicity of cervical cancer, and implicate that blockade of Axl signaling in cervical cancer might be an effective way to reduce the progression of cervical cancer.

73/B20

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Malignant tumors are composed of a small subset of distinct cancer cells, termed “cancer stem cells” (typically less than 5 - 15 % of total cancer cells based on cell surface marker expression), which have great proliferative potential, when compared to the more differentiated parental cancer cells. The cancer stem cells (CSC) have the potential of differentiating into their parental cancer phenotypes, which have very limited proliferative potential. Data have been provided to support the existence of cancer stem cells in several different types of cancer, including human blood, brain, prostate, ovarian, melanoma, colon, liver and breast cancers. In this study we have recently reported the identification of a subpopulation of pancreatic cancer stem cells that express the cell surface markers CD133+CD44+CD24+SSEA-1+TRA-1-61+& TRA1-81+ (0.5-2.0% of all human pancreatic parental cancer cells) that function as pancreatic cancer stem cells. The CD133+CD44+CD24+SSEA-1+Oct3/4+TRA-1-61+& TRA1-81+ pancreatic cancer stem cells are highly tumorigenic and possess the stem cell-like properties of self-renewal and the ability to produce differentiated progeny. Pancreatic cancer stem cells also demonstrate upregulation of SSEA3+,SSEA4+ upon differentiation into parental cancer phenotype. As for clinical importance, cancer stem cells have shown resistance to standard therapies and may play a role in treatment failure or disease recurrence. The cell based assay system for pancreatic cancer stem cell enables one to further their drug discovery signaling pathway mechanisms that regulate their growth and survival. This pancreatic stem cell based assay system may provide novel therapeutic approaches into treatment of pancreatic cancer patients, which are resistant to standard chemotherapy and radiation. The pancreatic cancer stem cell based assay system will provide a high through put screening of novel and potential drug candidates for the pancreatic cancer patients.
74/B21
Isolation of Cells with Characteristic of Stem/Progenitor Cells from Epithelial Ovarian Cancer.
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Several studies support the presence of putative cancer stem cells (CSC) in ovarian cancer (EOC), on the basis of sphere forming capability, presence of a side population (SP) (and expression of the CD133 marker. The chemokine receptor CXCR4, expressed by CSCs of different solid tumors, is frequently present on a subset of ovarian tumor cells, but not yet described as CSC marker in EOC. The present study aims to investigate whether, like CD133, also CXCR4 expressing cells could identify a subpopulation with characteristics of cancer stem/progenitor cells in human primary EOC and ascites. One of the hypotheses on the origin of CSC indicates the corresponding normal stem cells as potential source of the tumor counterpart.

By FACs analysis CD133 and CXCR4 show a variable degree of expression in OSE cells (early passage), ranging from 0.1-20% and from 50-70% of the cells, respectively. By real time PCR, both markers appeared up regulated in early In Vitro passages and then down regulated in later passages. In a wide range of serous ovarian tumors and ascites analyzed, the expression of CD133 and CXCR4 was restricted within a subpopulation of tumor cells. By immunohistochemistry of several EOC samples CD133 had a focal reactivity whereas by FACs analyses of a panel of EOC and ascites CD133 expression ranged between 0.1%-19,1% and 0.3%-34,3%, respectively. Moreover a variable degree of CXCR4 expression was found in primary EOC (from 7% to 70%) and ascites samples (from 3% to 65%). We are now focusing on the sorting separation of CXCR4 and CD133 positive subpopulation of ovarian tumor cells to investigate the possible relationship between the two populations. By serial In Vivo and In Vitro passages of ascitic cells derived from a poorly differentiated EOC positive for CD133 and CXCR4, both expressions were found to be maintained. Sorted CXCR4 or CD133 positive tumor cells were evaluated by Real Time PCR for stem marker (Oct4, Nanog CD133 and Nestin). The obtained results support the idea that ovarian CSC/TIC could arise from OSE stem cells and could be included in the subpopulation of CXCR4+/CD133+ cells.

75/B22
Correlation of Human Papillomavirus (Hpv) in Cervix Cancer, Leiomyoma, and Normal Samples.

Many studies have shown that high-risk human papillomavirus(HPV) is directly associated with development of cervix cancer. There are many types of high-risk HPV which are type 16, 18, 31, 33, 45 and so on. Many organs, such as heart, digestive duct, viscera organs, uterus, have myoma. Among those organs, leiomyomata of the uterus are common. but studies concerning leiomyoma have been less than those of HPV. Leiomyoma is a tumor caused in muscle tissue. So, in order to study about correlation of HPV and leiomyoma, we collected myoma, cervix cancer, and control samples from obstetrics and gynecology of Hospital of Gachon university of medical and science. DNAs were extracted from these samples. We practiced first PCR method using HPV common primer to amplify these DNAs and then we carried out second PCR method using HPV type 16 and 18 primer to amplify DNAs. Then PCR products were analyzed whether HPV type 16 and 18 exist in those samples or not. It is originally well known fact that cervix cancer is associated with HPV. So we will make an experiment in order to confirming that fact and investigating our expectation that HPV is related to myoma.
**76/B23**

**Primary Cilia and Aberrant Cell Signaling in Human Ovarian Cancer.**

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Ovarian cancer is the fourth leading cause of death among women in industrialized countries. Around 80-90% of these cancers stem from the ovarian surface epithelium (OSE). Yet the molecular mechanisms as to how the cancer arises are poorly understood. Here we investigated the relationship between primary cilia in cultures of normal human OSE cells isolated from 20-34 year old women and in human ovarian adenocarcinoma cell lines (OVCAR3 and SKOV3), which are derived from human OSE of ovarian cancer patients. Initially we show by immunofluorescence microscopy analysis that primary cilia protrude from normal OSE, and that about 80% of human OSE cells in culture form primary cilia after serum starvation to induce growth arrest. In contrast, only about 20% of OVCAR3 and SKOV3 cells were ciliated during growth arrest, indicating that defects in ciliogenesis are linked to ovarian cancer. Induction of quiescence was verified by dephosphorylation of retinoblastoma protein and nuclear loss of PCNA and Ki67, such that growth-arrested cells were e.g. Ki67 negative. In normal OSE cells, components in signaling pathways that are defective in tumor cell formation and invasion localized to primary cilia, including PDGFR\(\alpha\) and Hedgehog (Hh) components. Upregulation and activation of PDGFR\(\alpha\) was reduced in OSE cancer cells compared to normal OSE, whereas Hh signaling measured by Ptc-1 expression was greatly upregulated in cancer cells compared to normal cells. Preliminary data further suggest that inhibition of AurA - either by specific inhibitors or siRNA - stimulates ciliary formation in cancer cells, and this increase may be associated with partly restoration of the normal ciliary signaling pathways. These results support the model by which primary cilia are associated with malignant transformation of OSE that may represent a novel drug target with therapeutic potential in epithelial cancers. Further analysis will be required to understand the importance of AurA and its upstream regulators in ciliary disassembly and regulation of signalling pathways in malignant cell transformation.

**77/B24**

**Cell Stiffness Correlates with Invasive Potential in Ovarian Cancer: A Magnetic Tweezer Study.**

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In cancer, genetic changes produce phenotypes that have altered responses to signals specific to the tissue of origin, leading to increased proliferation, migration and invasion to surrounding tissues. The specific process of migration and invasion in the context of cancer progression relies on alterations in cell biomechanical properties including cell stiffness, morphology, cytoskeletal changes and alterations in signaling pathways that regulate the same. We hypothesized that during ovarian cancer progression alterations in the actin cytoskeleton resulting in increased invasiveness also result in a reduction in cell compliance/stiffness. To test the hypothesis we used ovarian cancer cell lines, and primary ovarian cancer cells obtained from ascites of patients with advanced stage disease and characterized their invasive potential using a transwell invasion assay, and simultaneously examined cell stiffness using the 3D Force Microscope (3DFM). The 3DFM produced ~100 pN pulses using 2.8 um fibronectin-coated beads. Force-response curves were analyzed for compliance and compared between cell lines. We found that there is a remarkable correlation between stiffness and invasion with the stiffest cell type being the least invasive and vice versa. Conversely, reducing invasiveness by expressing T\(\beta\)RIII, a previously demonstrated modulator of cell invasion and migration in non-expressing cancer cells reduced cell stiffness, thereby strongly correlating with reduction in invasive potential. Hence, we propose the presence of a distinct cytoskeletal and biomechanical signature of cancer cells during cancer progression that can be measured using 3DFM.
78/B25
Cellular and Molecular Mechanisms Involved in the Anti-Metastatic Effect of Pomegranate Juice in Prostate Cancer Cells.
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Prostate cancer is the most common invasive malignancy and the second leading cause of cancer-related deaths among U.S. males. Pomegranate juice from tree Punica granatum has been identified as a natural product that inhibits the growth and reduces the invasiveness of prostate cancer cells. However, the underlying mechanisms involved in the anti-metastatic effect of Pomegranate Juice remain unclear. We have recently found that, in addition to causing cell death of hormone-refractory prostate cancer cells, Pomegranate Juice also increases cell adhesion and decreases cell migration of the cells that do not die. We hypothesize that Pomegranate Juice inhibits the migratory and metastatic properties of hormone refractory prostate cancer cells by stimulating the expression and/or activation of molecules that alter the cytoskeleton and the adhesion machinery of the cell, resulting in enhanced cell adhesion and reduced cell migration. To test this hypothesis, we used both Affimetrix gene arrays to study gene expression and microRNA arrays to study the non-coding RNAs, which are molecules known to have profound regulatory effects on gene expression in normal cells and to be disregulated in cancer cells. We found that several genes involved in cell adhesion such as E-cadherin, CD44, and Myristoylated alanine-rich protein kinase c (MARCKS) were up-regulated and several genes involved in cell migration such as type I collagen, tenascin C and chimerin 1 were down-regulated after Pomegranate Juice treatment. Discovery of the mechanisms by which this enhanced adhesion and reduced migration are accomplished can potentially lead to more specific and effective treatments of the disease in particular when the components of the juice responsible for these effects are identified.

79/B26
Maintenance of Stem Cell Properties in Prostate Cancer-Derived CD133+MDR1+ Cells.
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CD133 (prominin-1), a 5-transmembrane glycoprotein, has recently been considered an important marker that represents the subset population of cancer stem-like cells. The purpose of the present study is to isolate cancerous stem-like cells from normal healthy volunteers and prostate cancer patients (CD133+) which also express MDR1 and to ascertain the influence of Oct-4 on ‘stemness’ and differentiation of these CD133+ cells towards epithelium. In the present study, CD133+ cells were isolated using magnetic beads from normal healthy volunteers and prostate cancer patients (NV-CD133+ and PC-CD133+). The isolated cells were analyzed using flow cytometry and western blot technique for CD133, MDR1 and Oct-4. CD133+MDR1+ cells were cultured in presence and absence of antihuman Oct-4 blocking antibody. Our results showed higher Oct-4 expression in PC-CD133+ cells with the ability to self-renew and may represent a reservoir with differentiation potential for generating prostate cancer cells. Furthermore, PC-CD133+ cells highly co-expressed the multiple drug-resistant marker MDR1. The treatment with Oct-4 blocking antibody can specifically block the capability of PC-CD133+ cells to differentiate into prostate epithelial cells bearing CD57. So finally we conclude that PC-CD133+ cells displayed a higher Oct-4 expression with the ability to self-renew and may represent a reservoir with differentiation potentials for progression of prostate cancer. The MDR1 expression of PC-CD133+ cells In Vitro and In Vivo is partially due to preferential activation of Oct-4 gene expression.

80/B27
Identification and Characterization of FKBP52-Specific Inhibitors for Prostate Cancer Treatment.
Introduction: Prostate cancer is a leading cause of death among African American males in USA. Androgens are the major stimulator of prostate tumor growth. Androgen-independent tumor growth during the late stage of the disease renders the current anti-androgen therapy futile since these treatments depend on androgen antagonists which compete with hormone for binding the Androgen Receptor (AR) hormone binding domain (HBD). Thus, drugs that target AR regulatory mechanisms and prevent prostate cancer cell growth are promising alternatives for the treatment of hormone-refractory prostate cancer (HRPC). The FK506-Binding Protein 52 (FKBP52) is proven to be an important regulator of AR receptor-mediated signal transduction in cellular and animal models and represents a potential target for treatment of HRPC. These studies are aimed at inhibiting endogenous androgen-dependent gene expression and FKBP52 association with the receptor complex. Methodology: A modified receptor-mediated reporter assay in yeast and mammalian cells was used to screen a natural compound library for FKBP52 inhibitors. AR-HBD-compound cocrystallographic structures were performed, as well as Surface Plasmon Resonance in order to determine the binding of the compounds to AR. Results: We identified molecules that efficiently and specifically inhibit FKBP52-mediated regulation of AR function by binding to a regulatory surface on the AR-HBD. We have demonstrated that these compounds do not compete with hormone for binding the HBD, nor do they compete with coactivator peptides. In addition, these compounds inhibit AR function by preventing hormone-dependent dissociation of the FKBP52-AR complex which results in low levels of hormone-bound receptor in the nucleus. Preliminary assays in LNCaP (prostate cancer) cells have demonstrated that the compounds inhibit Prostate-Specific Antigen expression and androgen-dependent proliferation. At the half-lethal dose (LD50) none of the compounds displayed cellular toxicity within the effective concentration range. Conclusion: We have identified molecules that inhibit AR function and represent an exciting new approach for HRPC treatment. In addition, these studies will lead to FKBP52-receptor interaction models.

81/B28
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Calcium transport proteins, including calcium ATPases, are responsible for maintaining cytosolic calcium homeostasis and calcium levels in intracellular organelles. Deregulation of global calcium homeostasis can modulate cellular proliferation. The Golgi-associated calcium ATPases maintain Golgi calcium levels via sequestration of cytosolic calcium into the Golgi secretory pathway. These ATPases may also influence global cellular calcium regulation by calcium sequestration and/or alterations in the post-translational modifications of proteins important in the regulation of intracellular calcium. We initially queried a microarray database of 295 clinical breast cancer samples for expression of Golgi-associated calcium ATPases and found differential expression amongst transcriptional breast cancer subtypes. To understand the role upregulated ATPases may have in breast cancer, ATPase expression was silenced in breast cancer cell lines. We used a low density siRNA transfection protocol and Dharmacon OnTarget plus Smartpool siRNA. Silencing (~95%) was confirmed 24 - 120 h post transfection using real time RT-PCR and resulted in a significant decrease in cellular proliferation. Altered cellular proliferation can be associated with the induction of endoplasmic stress (ER). However, in breast cancer cell lines, Golgi-associated calcium ATPase-mediated proliferation was independent of ER stress induction (tunicamycin 0.1 - 10 µg/mL) and Golgi-associated calcium ATPase silencing did not sensitize the cells to ER stress. To assess the role of Golgi-associated calcium ATPases on cytosolic free calcium regulation we used a FLIPRTETRA Imaging System. We examined the effects of siRNA on calcium responses initiated by cyclopiazonic acid and in the presence of different calcium mobilising agents (ATP, thrombin and trypsin). This study suggests that some breast cancers are...
associated with remodelling of calcium homeostasis through alterations in the transcription of Golgi-associated calcium ATPases. The aberrant overexpression of these calcium pumps, and their contribution to cellular proliferation, suggests these transporters may represent a novel and specific target for therapeutic treatment for some breast cancer subtypes.

82/B29
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We have identified the neural cell adhesion molecule L1 (L1-CAM, CD171) as a marker of “dedifferentiation” and mediator of malignancy in pancreatic ductal adenocarcinoma (PDAC). L1 has been described as a mediator of the malignant phenotype or indicator of poor prognosis in melanoma, breast, ovarian and colon cancer, with roles in regulating signaling and experimental metastasis, however previous reports on L1 expression in PDAC range from absent to high. Our data demonstrate that L1 is expressed in poorly-differentiated PDAC cells in situ. Moreover, our data demonstrate steady-state saturated phosphorylation of threonine-1172 (T1172) in the L1 cytoplasmic domain in PDAC cells In Vitro and in situ, in contrast to melanoma and neural cells. Although blockade of PKC and CKII activity caused T1172 dephosphorylation in PDAC cells, only CKII was capable of phosphorylating T1172 of recombinant L1 cytoplasmic domain in vitro. Importantly, L1 phosphorylation drives a change in the extracellular structure of L1, whereby membrane-proximal epitopes become obscured and distal epitopes become exposed, suggesting a shift from the previously described closed conformation to the open and putatively multimerized conformation. The multimerization of L1 has been demonstrated previously to regulate integrin binding, and we demonstrate that L1 bound to integrins αvβ3 and αvβ5 exhibits differential anti-L1 antibody epitope availability, suggesting that T1172 phosphorylation is involved in regulating these ectodomain conformations and associated integrin binding. We further demonstrate that phosphorylation of T1172 is required for the ADAMs-mediated shedding of the L1 ectodomain that drives tumor cell migration. These data define a specific role for T1172 in regulating L1 activity in pancreatic ductal adenocarcinoma cells.

83/B30
miR-200c Modulates EGFR Signaling by Targeting Negative Feedback Regulator MIG-6/ERRFI1 in Breast Cancer.
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Fine-tuning of epidermal growth factor receptor (EGFR) signaling is crucial for maintaining cellular homeostasis and preventing unrestrained proliferation. Several negative feedback mechanisms have been shown to play major roles in this process. Mitogen-inducible gene 6 (MIG-6) is a central feedback inhibitor of kinase-active ERBB receptors and it is found to be down-regulated or lost in several human tumors, indicating a tumor-suppressor function. MicroRNAs are a class of small regulatory RNAs, which down-regulate their target genes by destabilization of the mRNA or repression of translation. Several miRNAs have been shown to be overexpressed in tumors, exerting an oncogenic function by suppressing tumor-suppressor genes. This study aimed at identifying oncogenic miRNAs targeting MIG-6, thus enhancing mitogenic signaling, to represent them as therapeutic targets. Whole genome miRNA/mRNA expression profiles followed by qRT-PCR and Western blot experiments in different breast cancer cell lines identified miR-200c as the most probable regulator of MIG-6 out of all predicted miRNAs. Ectopic expression of miR-200c in MDA-MB-231 breast cancer cell line substantially decreased MIG-6 protein level. Noteworthy, luciferase reporter assay revealed MIG-6 as a direct target of miR-200c. MDA-MB-231 cells were shown to be more responsive to EGF stimulation after miR-200c overexpression or MIG-6 knockdown, reflected by increased and more sustained phosphorylation of ERBB2 and its downstream kinase AKT. Additionally, miR-200c could notably increase proliferation of cells stimulated with EGF as reflected with higher percentage of cells in S and G2/M phase of cell
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cycle. As miR-200c has been known to regulate epithelial-mesenchymal transition (EMT) by targeting the TGF-β induced E-cadherin repressors ZEB1 and ZEB2, our results could implicate miR-200c as a key regulator of the cell’s decision between migration and proliferation. Further study will include analysis of breast cancer patient samples for miR-200c and its targets MIG-6/ZEB1/ZEB2 as well as the possible role of miR-200c in resistance against ERBB receptor-targeted drugs e.g. gefitinib and trastuzumab.

84/B31 ABSTRACT WITHDRAWN

85/B32

Breast cancer is the most common malignancy among women in developed countries, affecting more than a million women per year worldwide. Of these, triple negative breast carcinoma represents 10-17%. Triple negative breast carcinomas, characterized by estrogen, progesterone and HER2 receptor negativity are very aggressive tumors with poor prognosis. Breast Cancer Stem derived from triple negative parental Breast Cancer tumors, are a subpopulation of cells within the parental breast cancer population within the individual which are positive for the following markers: CD133+CD44+CD24+ESA+SSEA-1+TRA-1-61+ & TRA1-81+ and Oct ¾ these Breast Cancer Stem cells are highly tumorigenic and possess the stem cell-like properties of self-renewal and the ability to produce differentiated progeny. Breast Cancer Stem cells also demonstrate up regulation of SSEA3+,SSEA4+ upon differentiation into parental cancer phenotype. Individualized treatment (tailored therapy) based on molecular biology markers of tumor and patient is the trend in clinical practice these days. However, molecular targets and predictors for the treatment of triple negative breast carcinoma do not currently exist. With the identification and characterization of Breast Cancer Stem Cells from parental triple negative tumors, enables one to screen novel drug candidates for potential development of therapeutics for triple negative Breast Cancer Patients. In this study we have utilized Breast Cancer Stem Cells from triple negative Tumors to screen potential drug candidates. The Breast Cancer Stem Cell based assay system may provide novel therapeutic approaches into treatment of triple negative breast cancer patients, which are resistant to standard chemotherapy and radiation.

86/B33
The Role of Znf217, a Gene Amplified in Breast Cancer, during Neoplastic Progression and Targeted Drug Therapy.
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The 20q13 region of the human genome is highly amplified in 20-30% of early stage human breast cancers, and this amplification correlates with poor prognosis. Several groups identified the human ZNF217 transcription factor gene as one of two candidate oncogenes in 20q13.2 that correlates with breast cancer and gastric adenocarcinomas, and overexpression of ZNF217 leads to immortalization of human mammary epithelial cells. We find that high ZNF217 expression correlates with poor survival in human breast tumors. In addition, patients with basal subtype primary tumors have lower expression levels of ZNF217 than do patients expressing other breast cancer subtypes. To further characterize downstream pathways affected by ZNF217 in breast cancer progression, we overexpressed mouse Znf217 in mouse primary cells and immortalized mouse mammary epithelial cell lines. Microarray expression profiling in these cells identified a number of downstream genes including other genes found in human 20q13.2, genes repressed by transcriptional repressor complexes known to be associated with Znf217, genes that play a
role in differentiation in other tissues, and genes that play a role in stem cell expansion. If ZNF217 is a target gene in 20q13.2 driving breast cancer progression, then it is a candidate for targeted drug development. We identified drugs from the Developmental Therapeutics database that have the best correlation in NCI60 tumor cell lines between their IC50s and increased ZNF217 expression levels. To validate the drug that best correlated with cell death in overexpressing ZNF217 cell lines, we tested it on human breast tumor cells with low level expression of ZNF217. Cells overexpressing ZNF217 become resistant to doxorubicin treatment, but treatment with our candidate ZNF217 inhibitor overcomes the ZNF217-induced resistance to doxorubicin-induced cell death. Therefore, ZNF217 is a potential drug target for breast cancer.

87/B34

**Human HER2+ Breast Cancer Stem Cells Utilized for Drug Discovery Therapeutics for HER2+ and Estrogen & Progesterone Negative Breast Cancer Patients.**


Breast cancer is the most common malignancy among women in developed countries, affecting more than a million women per year worldwide. The HER2+, Estrogen and Progesterone negative breast carcinoma is characterized by estrogen, progesterone receptor negativity and HER2 receptor positive are a sub-classification of HER2+ breast Cancers. Breast Cancer Stem derived from HER2+ and double negative parental Breast Cancer tumors, are a subpopulation of cells within the parental breast cancer population within the individual which are positive for the following markers: CD133+CD44+CD24+ESA+SSEA-1+TRA-1-61+& TRA1-81+ and Oct ¾ these Breast Cancer Stem cells are highly tumorigenic and possess the stem cell-like properties of self-renewal and the ability to produce differentiated progeny. Breast Cancer Stem cells also demonstrate up regulation of SSEA3+,SSEA4+ upon differentiation into parental cancer phenotype. Individualized treatment (tailored therapy) based on molecular biology markers of tumor and patient is the trend in clinical practice these days. However, molecular targets and predictors for effective treatment of HER2+ double negative breast carcinoma do not currently exist. With the identification and characterization of Breast Cancer Stem Cells from parental HER2+ double negative tumors, enables one to screen novel drug candidates for potential development of therapeutics for HER2+ double negative Breast Cancer Patients. In this study we have utilized Breast Cancer Stem Cells from HER2+ double negative Tumors to screen potential drug candidates. The Breast Cancer Stem Cell based assay system may provide novel therapeutic approaches into treatment of HER2+ double negative breast cancer patients, which are resistant to standard chemotherapy and radiation.

88/B35

**Estrogen Activates G-Protein Inwardly Rectifying Potassium Channels (GIRK) in MCF-7 Breast Cancer Cells.**

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Breast cancer is the second leading cause of cancer death. Previous work from our laboratory has indicated the importance of G-protein inwardly rectifying potassium channels (GIRK) in breast cancer. Estrogen, a major risk factor for breast cancer, has been correlated with potassium channel function in the brain. Accumulating evidence has shown that many of these ion channels are essential for cell proliferation and cell survival, which are key events in cancer progression. In the present study, we investigated the chronic effects of estrogen in MCF-7 breast cancer cells. We found a dramatic increase in GIRK1 and GIRK2 protein levels in response to 10 nM estrogen treatment, as well as an increase in intracellular potassium accumulations and increased cellular proliferation after 24-48 hours of treatment. Protein levels were monitored by western blotting, proliferation was measured by 5-Bromo-2’ deoxy-uridine (BrdU) assay, and PBFI potassium-specific florescent dye was used to measure potassium accumulation. GIRK1 RNA interference knockdown decreased estrogen receptor alpha protein levels and activation. In addition, estrogen treatment resulted in increased phosphorylation of specific members of G-protein couple receptor
(GPCR) and MAPK signaling pathways, which have been shown to be responsive to GIRK1 knockdown by our laboratory in MDA-MB-453 breast cancer cells. Our data indicates that GIRK1- and GIRK2-specific channels are the predominant functional subunits in MCF-7 cells. Our data also links GIRK function to the major breast cancer risk factor of estrogen and suggest that GIRK may have relevance as a therapeutic target, especially in concert with already approved treatment options such as anti-estrogen and anti-aromatase compounds.

89/B36
Effects of PKCδ and PLC-γ1 on MCP-1 Expression in Taxol-Induced Breast Cancer Cell Death.
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Monocyte chemotactic protein-1 (MCP-1) is a CC chemokine that plays an important role in immune cell migration. It has been reported that chemokines including MCP-1, are involved in angiogenesis and metastasis. However, the exact role of chemokines in cancer development is still obscure. We investigated the involvement of MCP-1 in taxol-induced breast cancer cell death. The anti-cancer drug taxol induced MCF-7 breast cancer cell death. Treatment with taxol increased the mRNA expression level of MCP-1 in a dose and time dependent manner. Up-regulation of MCP-1 by taxol was augmented in cells treated with rottlerin, a specific inhibitor of protein kinase Cδ (PKCδ). In addition, taxol-induced MCP-1 expression was reduced by the ectopic expression of PKCδ in a dose dependent manner, indicating that PKCδ plays a negative role in taxol-induced MCP-1 expression in MCF-7 cells. On the other hand, taxol-induced up-regulation of MCP-1 was reduced in cells treated with U73122, an inhibitor of phospholipase C (PLC), and ectopic expression of PLC-γ1 increased the expression of MCP-1 in taxol-treated MCF-7 cells, indicating PLC-γ1 functions as a positive regulator in taxol-induced MCP-1 expression. These results indicate that MCP-1 is involved in taxol-induced breast cancer cell death and we propose that taxol induces up-regulation of MCP-1 by affecting both positive and negative regulatory signaling pathways.

90/B37
Rho GTPases in Disparate Effects of Soy Isoflavones on Breast Cancer Metastasis.
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Breast cancer is a major cause of death from cancer. Death from breast cancer is usually due to metastasis. The role of soy foods in breast cancer prevention is known but their effects on metastatic breast cancer remain to be understood. The objective of investigating a role for soy isoflavones in established breast cancer was achieved by testing the effect of the major soy isoflavones on progression of mammary tumors in nude mice created from green fluorescent protein (GFP) tagged-MDA-MB-435 bone metastatic variant. Following tumor establishment, mice were orally gavaged with vehicle or soy isoflavones: genistein (10 mg/kg body weight (BW)), daidzein (10 mg/kg BW), or genistin (50%), daidzein (40%), and glycitein (10%) three times per week. Tumor progression was quantified by whole body fluorescence image analysis twice a week followed by microscopic image analysis of excised organs for metastases. Results show that daidzein significantly increased while genistein decreased mammary tumor growth by 38% and 33% respectively, compared to vehicle. Daidzein increased lung, heart, and kidney metastases while genistein decreased bone and liver metastases. Combined soy isoflavones did not affect mammary tumor growth but increased metastasis. Phosphoinositide-3-kinase (PI3-K) pathway real time PCR array analysis on excised tumors demonstrates that genistein significantly downregulated 26% of the genes tested, including Rho GTPases ROH1, RAC1, and CDC42, and their downstream effector PAK1. Daidzein upregulated 14% of the genes including CCND1, GRB2, MAPK1, JUN, CTNNB1, IRS1, EIF4G1, and GSK3B that regulate proliferation and protein synthesis. Western blotting of mammary tumor extracts or breast cancer cell lysates following genistein or daidzein treatment show that genistein downregulated RhoA, Rac1, and Cdc42 while
daidzein upregulated Rac1 protein expression. Differential regulation of Rho GTPases may account for the disparate responses of breast cancers to genistein and daidzein diets. Thus, soy diets may exert complex effects on breast cancer patients and survivors. This research was supported by grant numbers AICR IIG 03-31-06 and NIH/NIGMS SC3GM084824 to SD and NCCR/NIH 2G12RR003035 and S06GM050695 to UCC.

91/B38
How Does MenaINV Sensitize EGF Receptor to EGF?
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Invasive breast tumor cells have been shown to uniquely express the MenaINV isoform of Mena. MenaINV has been shown to enhance the sensitivity of breast tumor cells to EGF and correspondingly, increased metastatic potential. To determine the molecular mechanism that mediates the MenaINV-elicited increase in sensitivity to EGF, we studied the localization and dynamics of the EGF receptor (EGFR) in MenaINV expressing tumor cells. Using TIRF imaging of E11 cells (MTLn3 cells stably transfected with EGFR-GFP) transiently transfected with mCherry-MenaINV, we found that EGFR and MenaINV colocalize at focal adhesions. Consistent with this observation, EGFR-GFP also colocalized with mCherry-Paxillin, a focal adhesion component. When EGFR clusters were counted in E11 cells with or without mCherry-MenaINV expression, cluster numbers were found to be independent of mCherry-MenaINV, but increased along with the increased EGFR expression levels. In addition, fluorescence recovery after photobleaching (FRAP) measurements of EGFR-GFP dynamics in E11 cells revealed no differences in recovery half time (15 sec) and mobile fraction (60%) that correlated with mCherry-MenaINV expression. Therefore, while EGFR and MenaINV colocalize at focal adhesions, EGF clustering and dynamics in the plasma membrane are not affected by MenaINV expression in cells that overexpress the EGFR. MenaINV may increase sensitivity to EGF through interactions with a subpopulation of EGFR that is in focal adhesions. Nevertheless, it remains possible that clustering of EGFR in sub-resolution-clusters (<250nm) in the plasma membrane that are not in focal adhesions contribute to the increased EGFR sensitivity observed in cells expressing MenaINV. Initial EM data indeed suggests that EGFR clusters in small domains.

92/B39
Benzyl Isothiocyanate Inhibits Metastasis of Breast Cancer Cells In Vitroand In Vivo.
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Benzyl isothiocyanate (BITC) is a hydrolysis product of glucotropaeolin found in cruciferous vegetables, and has been reported to have anti-cancer properties. To examine the effect of BITC on metastatic capacities of breast cancer cells in vitro, MDA-MB-231 human and 4T1 mouse breast cancer cells were cultured in the presence of 0 - 4 μmol/L with or without 10 ng/mL hepatocyte growth factor (HGF). BITC inhibited basal and HGF-induced cell migration, invasion, and adhesion in dose-dependent manners in both MDA-MB-231 and 4T1 cells. However, BITC did not affect the viability of these cells at these concentrations. BITC decreased basal and HGF-stimulated secretion and activity of urokinase-type plasminogen activator (uPA) in MDA-MB-231 cells. By contrast, BITC increased protein expression of plasminogen activator inhibitor-1 in MDA-MB-231 cells. BITC decreased basal and HGF-induced secretion of tissue inhibitor of metalloproteinase (TIMP)-1, but increased TIMP-2 secretion by MDA-MB-231 cells. However, BITC did not affect secretion of matrix metalloproteinase (MMP)-2 or MMP-9. Furthermore, BITC inhibited HGF-stimulated phosphorylation of cMet and Akt, and reduced basal and HGF-stimulated translocation of p65 to the nucleus and NF-κB DNA binding activities in MDA-MB-231 cells. To examine whether BITC inhibits cancer metastasis in vivo, 4T1 cells (50,000 cells) were injected into the inguinal mammary fat pad of syngeneic female BALB/c mice. One day later, the
mice were divided into 3 groups and subjected to gavage with 0 - 10 mg BITC/kg body weight/day for 4 weeks. Oral administration of BITC significantly decreased the numbers of pulmonary tumor nodules and total pulmonary metastatic volume. The present results indicate that BITC has potential as an anti-metastatic agent for breast cancer patients.

93/B40
Her2 and K-RasG12D Dependent Centrosome Regulatory Targets Are Critical to Centrosome Amplification in Mammary Epithelial and Breast Cancer Cells.
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Over 80% of breast cancers and ductal hyperplasias harbor elevated frequencies of centrosome amplification (CA), a process leading to defective and multipolar mitotic spindles, aneuploidy and chromosome instability. To date, the signaling pathways that generate CA in breast cancers remain a mystery. We hypothesized that Her2, Ras and c-Myc, proto-oncogenes constitutively active in breast cancers and which oncogenic versions trigger mouse mammary cancers, initiate mammary cancers by inducing CA and chromosome breaks, precursors to chromosome instability. To test this hypothesis, we selectively expressed K-RasG12D and c-Myc in mouse mammary epithelial cells for five days using the MMTV-rtTA; tetO inducible transgenic model. Addition of doxycycline to the diet of K-RasG12D and/or c-Myc transgenic mice resulted in elevated expression of the oncogenes and various degrees of hyperplasia and dysplasia; K-RasG12D exhibiting more severe dysplasia. Both K-RasG12D and c-Myc led to increased S phase fractions, apoptosis and double strand breaks. Remarkably, only K-RasG12D resulted in elevated frequencies of CA. K-RasG12D exclusively induced expression of cyclin D1, while K-RasG12D and c-Myc led to over-expressed Nek2A. Abolishing expression of cyclin D1, Cdk4, and Nek2 in MCF10A mammary epithelial cells abrogated H-RasG12V-induced centrosome amplification. This suggests that H-RasG12V plays an important role in CA. To evaluate the clinical relevance of our results, we assessed whether breast cancer cells over-expressing Her-2 resulted in CA. We showed that these cancer cells harbor CA relative to MCF10A cells. These cells also show Nek2A, Plk-4, p27, cyclin D1, E2F3a, and E2F1 upregulation. siRNA knockdown of Cdk4, cyclinD1’s catalytic partner, greatly reduces CA in Her-2 over-expressing cells. These results suggest that the Her-2/Ras pathway is critical to the induction of CA in both mouse and human mammary cancers.

94/B41
Circulating Tumor Cells in Primary Breast Cancer.
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Objective: Circulating tumor cells (CTCs) are considered to be responsible for cancer metastasis in the distant organs. Elucidating mechanisms underlying CTC release will lead to a new strategy for cancer treatment. CTCs in metastatic breast cancer have been reported to reflect status of systemic cancer activity. However, CTCs in primary breast cancer remain to be studied. In this study, we examined CTCs in primary breast cancer patients to elucidate characters of CTCs in primary breast cancer. Methods: Forty-five primary breast cancer patients before preoperative systemic chemotherapy were enrolled in this study. CTCs were assessed in 7.5ml blood by the immunomagnetic detection system, CellSearch system (Veridex, LLC, NJ). CTCs were defined by the phenotype, Ep-CAM+, CK8/18/19+, DAPI+, CD45-. Viable and apoptotic CTCs were separated using FITC-M30 antibody (PEVIVA, Sweden). Results: Thirty five percents of the patients showed more than 2 CTCs in 7.5ml blood. When the patients were categorized by the intrinsic subtypes, patients with HER2 subtype cancers showed a higher number of CTCs than the other subtypes (p < 0.01). There was no difference in the ratio between viable and apoptotic CTCs among cancer subtypes. We analyzed CTC count in association with the response to preoperative systemic chemotherapy. There was no correlation between CTC count and tumor response. However, higher rates of apoptotic CTCs were associated with better response to the treatment (p = 0.02), suggesting that apoptotic CTC rates may predict treatment response to chemotherapy. Conclusions: There was a significant difference in CTC count among intrinsic
subtypes of breast cancer, which may explain the difference in clinical outcome of breast cancers with different intrinsic subtypes. Separating viable and apoptotic CTCs may be useful to predict treatment response to chemotherapy.

95/B42
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Production of vascular endothelial growth factor C (VEGF-C), a most potent lymphangiogenic factor, by normal and cancer cells can be linked to the activation of Toll-like receptors (TLRs) because the promoter region of the gene contains the putative NF-κB binding site. To test this hypothesis, we examined the effect of TLR2 ligand chitin on VEGF-C production by MDA-MB-231 human breast cancer cells, which have been recently reported to secrete constitutively high levels of VEGF-C in cell culture medium (Timoshenko et al, 2006) and to express abundantly TLR2 (Xie et al, 2009). The 80-90% confluent cell monolayers were treated with serum-free DMEM containing either chitin hydrolysate at serial dilutions corresponding 0.25-8 mM of total N-acetylglucosamine (GlcNAc) or NaCl (13.75-220 mM) for 24 h and the concentration of VEGF-C was measured in cell culture media using a solid phase sandwich ELISA kit. Cells treated with GlcNAc-specific lectin wheat germ agglutinin (WGA) were used as a positive control. Chitin hydrolysate was found to induce an immense dose-dependent increase in VEGF-C accumulation in cell culture medium of MDA-MB-231 cells, which was at a chitin GlcNAc concentration of 4 mM up to 10 folds more than the basal production. A moderate increase of VEGF-C secretion was also observed in the presence of hypertonic doses of NaCl, which mimicked the matrix of chitin hydrolysate stock solution, and in the presence of WGA. When chitin hydrolysate was added to cells together with WGA with an intention to inhibit the lectin effect, the overproduction of VEGF-C was sustained indicating that WGA-independent receptors were responsible for chitin-mediated stimulation of VEGF-C synthesis. To the best of our knowledge, this is the first study demonstrating that chitin hydrolysate serves as a strong inducer of VEGF-C synthesis by breast cancer cells activating most likely TLR2-mediated signaling pathway.

96/B43
Effects of Canola Oil on Breast Cancer Cell Growth and Multi-Drug Resistance.
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Canola oil, a naturally well balanced omega-6/omega-3 containing oil, has a very low level of saturated fat and relatively high levels of monounsaturated and polyunsaturated fatty acids. The beneficial effects of alpha-linolenic and oleic acids in canola oil are widely accepted in human health, particularly cardiovascular disease, but limited data are available regarding cancer, including breast cancer. We previously reported that canola oil has anti-proliferative effects on the cell growth of estrogen positive human breast cancer cells in vitro. The objective of the study was to investigate the effects of canola oil on breast cancer cell growth and multi-drug resistance. Three estrogen receptor positive breast cancer cell lines T47D, MCF7, MCF7/doxorubicin-resistant were cultured with media containing various combinations of canola oil and anti-estrogen drug tamoxifen or fatty acid synthase inhibitor cerulenin. Cell proliferation and caspase-3 activity were measured (1-4 days). Canola oil significantly inhibited the growth of both T47D and MCF7 cancer cells with tamoxifen (28.6% and 6.3% respectively) and cerulenin (25.1% and 6.5% respectively) on day 4, compared with tamoxifen or cerulenin alone. Canola oil also showed increased drug susceptibility on anti-cancer drug doxorubicin multi-drug resistant cells. Canola oil decreased the cell growth of MCF7/doxorubicin-resistant cells (8.9%) and increased the expression of caspase-3 activity (16.7%) on day 4, one of the crucial enzymes regulating the apoptosis pathway. The consistent and significant inhibition of canola oil, on the growth of these cancer cells appears to be due to increased apoptosis. Canola oil leads to breast cancer cell apoptosis, acts synergistically with anti-cancer drugs, and alleviates multidrug resistance. These results could be useful in the development of improved nutritional strategies for drug resistant breast cancer.
97/B44
Extracellular Proton Promotes the Migration of Breast Cancer Cells through the Activation of the Proton Sensing Receptor, oGPCR X.
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G-protein-coupled receptors (GPCRs) are the largest family of cell-surface molecules that regulate many cell functions. Recently, several GPCRs have emerged as critical players in tumor growth, angiogenesis and metastasis. Of 800 GPCRs, a half of them are orphan GPCRs, which has no known ligands or functions. Orphan GPCRs is a highly active area of research that already led to the identification of many ligands for previously orphaned GPCRs. However, little is known about the tumor-specific expression profiles and their roles in specific human cancers. Here, we profile expression of orphan GPCRs in breast cancer which is the most common malignant disease in women world. We selected 90 orphan GPCRs as targets and performed quantitative PCR analysis in non-transformed breast derived cell (MCF 10A) and breast cancer cell (MCF7 and MDA-MB 231). As a result, we newly found an orphan GPCR, oGPCR X is highly up-regulated in breast cancer cell and breast cancer tissues, suggesting that oGPCR X may be involved in the breast cancer progression and metastasis. To elucidate functions, we down-modulate the expression of oGPCR X in highly malignant breast cancer cell (MDA-MB-231) by using shRNA-Lenti virus system. Upon acid stimulation, oGPCR X did not effect on breast cancer cell proliferation. In contrast, acid-induced breast cancer cell migration was significantly impaired by oGPCR X knockdown cell lines compared with wild-type cell lines on acidic condition. Take together, our results suggest oGPCR X is aberrantly over-expressed in breast cancer and promotes acid-induced breast cancer cell migration. In addition, we expect that these novel finding provide a foundation for further studies for identifying the functions of novel orphan GPCRs in breast cancer.

98/B45
The Role of Genetic Polymorphisms of DNA Repair Gene RAD23B and Breast Cancer Risk in Puerto Rican Women.
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Breast cancer is the most commonly diagnosed cancer among women in the United States and Puerto Rico. Susceptibility to breast cancer is influenced by several factors, including age, DNA repair capacity and genetics. Rare high penetrance mutations in the breast cancer genes BRCA1 and BRCA2 only explain a small number of breast cancer cases. Low penetrance mutations in other DNA repair genes may confer higher risks of developing the disease in the general population. Previous studies have linked polymorphisms in the DNA Repair gene RAD23B with laryngeal cancer, lung cancer and breast cancer susceptibility. Our objective was to assess the contribution of single nucleotide polymorphisms (SNP) in RAD23B to breast cancer risk in Puerto Rican women. We genotyped three SNPs of the RAD23B gene rs10521083, rs10739234 and rs1805329 using the TaqMan PCR assay. Allelic and genotype frequencies were compared between 152 breast cancer cases and 251 controls recruited through breast surgery and oncology practices in Puerto Rico. Allele and genotype frequencies at rs10521083 and rs10739234 were not associated with breast cancer risk. However, the T allele at rs1805329 was more frequent in women with breast cancer than in healthy women (X2= 6.723, p<0.01). In conclusion, we provide evidence that genetic variants in the nucleotide excision repair gene RAD23B are associated with a higher risk of breast cancer in Puerto Rican women. Knowledge of the genetic basis to breast cancer susceptibility is likely to impact on personalizing breast cancer screening and preventive strategies.
Grape Polyphenols Inhibit Breast Cancer Progression through Regulation of the PI3K/Akt Pathway.
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Grape and red wine polyphenols have been widely studied due to their cancer preventive properties. However, much of these studies have focused on individual polyphenols at concentrations too high to be achieved via dietary consumption. We recently reported that combined grape polyphenols resveratrol, quercetin, and catechin (RQC) at physiologically relevant concentrations induced apoptosis and inhibited breast cancer cell proliferation, cell cycle progression, cell migration, and primary mammary tumor growth and metastasis to bone and liver (Schlachterman et al., 2008. J. Translat. Oncol. 1:19-27; Castillo et al., 2009. Clin. Exp. Met. 26:505-516). Herein, we used human phospho-kinase antibody arrays and Western blot analyses to investigate the molecular mechanisms of RQC action on breast cancer. Phosphorylation levels, and thus the activity, of Akt and its downstream effector endothelial nitric oxide synthase (eNOS) were decreased in breast cancer cells treated with 5µM RQC. Western blotting of excised tumors from mice following dietary RQC treatment showed an increase in phospho and total levels of IκBα, i.e. the inhibitor of the Nuclear Factor kappa-B (NFkB). Akt is activated by phosphatidylinositol 3-kinase (PI3K) and plays a central role in tumor development by inducing cell proliferation and survival pathways. eNOS also inhibits apoptosis and induces cell proliferation, angiogenesis, invasion, and metastasis. Moreover, the PI3K/Akt pathway activates NFkB, a transcription factor commonly hyperactivated in cancer to promote cancer cell survival and proliferation, that has been specifically implicated in cancer metastasis to the bone. Taken together, these results suggest that RQC treatment inhibits breast cancer progression and site-specific inhibition of metastasis to the bone by downregulation of Akt signaling to eNOS and NFkB. This study was funded by AICRIIG 03-31-06 and DoD BCRP W81XWH-07-1-0330 to S.D.; NIH/RCMI 2G12RR003035 to UCC; NIH/RCMI G12-RR03051 to UPR; and DOD BCRP W81XWH-08-1-0258 to LCP.

A Potential Mechanism for Omega 3 Polyunsaturated Fatty Acids to Inhibit Growth of Breast Cancer: Alteration of Location of G Protein in Plasma Membrane Microdomains (Lipid Rafts).
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The omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), inhibit the growth of human breast cancer cells. In order to explore the modulation of breast cancer cell growth by omega-3 fatty acids, we observed the effects of EPA and DHA on proliferation of MCF-7, the human breast cancer cell line. Three days treatment of MCF-7 cells with EPA and DHA inhibited cell proliferation, and DHA significantly decreased proliferation of MCF-7 cells about 25% compared to control group. Furthermore, activation of the G protein coupled estrogen receptor, GPR30, increased the proliferation of MCF-7 cells around 15% compared to control group. This was attenuated by DHA treatment. Previous studies suggested translocation of activated Gαs into lipid rafts (allen et al, 2005). DHA and EPA treatment reduced lipid rafts association of Gαs in MCF7 cells. Fluorescence recovery photo bleaching showed that omega-3 fatty acids treatment reduced the movement of Gαs on cell plasma membrane. These results give rise to the possibility that altered subcellular localization of Gαs and the subsequent change in Gαs signaling may be one mechanism whereby omega 3 fatty acids decrease breast cancer cell proliferation. (Supported by DOD)F

Twist: A Potential Novel Therapeutic Target to Prevent Breast Cancer Metastasis.
The transcription factor, Twist, is a crucial regulator of epithelial-mesenchymal transition (EMT) during embryogenesis and tumorigenesis, regulates breast cancer cell invasion and metastasis, and it is highly expressed in malignant breast tumors. Our objective is to design dominant-negative (DN) Twist mutants to inhibit wild type Twist activity and reduce Twist-mediated invasion of breast cancer cells. Deletion of the last 20 C-terminal amino acids ($\Delta$183-202) resulted in a non-functional Twist protein, as shown by lack transcriptional activity. Amino acid substitutions, S144K/R145E, did not result in lack of transcriptional activity, whereas a triple mutation, S144K/R145E/$\Delta$183-202, resulted in a further decrease of transcriptional activity, compared to $\Delta$E183-202 only. Moreover, the $\Delta$183-202 and S144K/R145E/$\Delta$183-202 mutants, acted as DNAs, as indicated by their ability to inhibit the transcriptional activity of wild type Twist. The mutants were also tested for their ability to inhibit Twist-mediated IL-8 production and migration/invasion of the Twist-expressing BT-549 breast cancer cell line in vitro. All three mutants, as well as silencing of Twist expression with shRNA, resulted in decreased IL-8 release. However, only the $\Delta$183-202 deletion resulted in decreased In Vitro invasion. Transcription factors are difficult targets of therapy due to their nuclear localization. To facilitate transfer into the nucleus of target cells, the mutants were fused to VP22, a protein transduction domain, which possesses membrane translocation properties. The VP22:Twist mutants will be tested in the same way as the Twist mutants and used to produce adenovirus carrying the transgene under the Tet-response element (TRE). Neural stem cells (NSCs), which naturally home to tumor sites, will be transduced and injected into tumor bearing mice. Once the stem cells have arrived to the tumor sites, the mice will be injected with doxycycline for VP22:DN Twist expression, which should be released from NSCs and taken up by target cancer cells. Given the role of Twist as a master regulator of breast cancer metastasis, delivery of DN Twist to tumor sites represents a promising therapeutic strategy to prevent breast cancer metastasis.

102/B49
NEDD9 Is Required for Activation of Oncogenic Signaling Pathways in Mammary Tumor Development.

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Neural precursor cell-Expressed Developmentally Downregulated gene 9 (NEDD9; HEF1, CASL) is a scaffolding protein regulating mitosis, survival, and migration. within the last 3 years, NEDD9 has been implicated in the progression and metastasis of several cancers. In addition, NEDD9 interacts with and positively regulates activity of key oncogenic signaling proteins such as Aurora-A kinase (AURKA) and Src. Recently, a viable and fertile Ned9 knockout strain has been developed that enables evaluation of the In Vivo role of NEDD9 in cancer initiation and progression. Interestingly, loss of Ned9 significantly limits age at tumour formation in the MMTV-Polyoma Virus middle T antigen (PyV(m)T) model of mammary tumorigenesis (Izumchenko, et al. Cancer Res., In Press.). In MMTV-PyV(m)T mice, there are few differences in invasion and apoptosis between Ned9 genotypes, but early tumors of MMTV-PyV(m)T;Ned9/- tumors had reduced markers of proliferation compared to MMTV-PyV(m)T;Ned9+/+ mice. Further, tumors harvested from MMTV-PyV(m)T;Ned9/- mice had generally reduced activation of key oncogenic signaling proteins, with the lowest levels of activation correlating with the greatest delays in tumor onset. To explore the role of NEDD9 in a second highly physiological model of mammary tumorigenesis, Ned9/- mice were crossed to MMTV-neu (HER2; ErbB2) transgenic mice, and compared with MMTV-neu;Ned9+/+ counterparts. Strikingly, by 18 months, 88% of MMTV-neu;Ned9+/+ mice developed tumors, in contrast to only 18% of MMTV-neu;Ned9/- mice. This indicates a novel role for NEDD9 in the initiation of mammary tumorigenesis. This is in contrast to the role of NEDD9 in progression and metastasis that is largely ascribed to NEDD9 cooperation...
with oncogenes focal adhesion kinase (FAK) and Src and indicates the relationship between NEDD9 and other oncogenic partners may be important in mammary tumor initiation.

**Metabolic Diseases I (103 – 124)**

**103/B50**

**Modulatory Effects of Adipocyte-Conditioned Media on Calcium Homeostasis in Isolated Cardiac Ventricular Myocytes.**

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Bioactive factors secreted by adipocytes (adipokines) play an important intermediary role in communication with many other cell types. However, effects of adipokines on cardiac myocyte electrophysiology and function are largely unknown. We have used a combination of optical as well as the whole-cell patch-clamp technique to examine the effects of adipocyte-conditioned media on myocyte contraction and calcium homeostasis in adult rat cardiac myocytes. Ventricular myocytes were isolated and plated on laminin-coated cover slips. To test the effects of adipokines, myocytes were cultured in adipocyte-conditioned media (ACM) for 24 hours. for control experiments, myocytes were cultured in non-conditioned media (NCM) or in HEK293 cell-conditioned (HCM) media. After 24 hours in culture (ACM, HCM or NCM), myocytes were loaded with a fluorescent calcium indicator (fluor-4AM, 10 µM) for simultaneous measurements of intracellular calcium transients and cell shortening. Cells were initially field stimulated at a low frequency (0.5 Hz, 40 V, 37°C) and only myocytes that showed no spontaneous contractions were studied. Compared to the control conditions (NCM or HCM), myocytes in ACM demonstrated a higher incidence of spontaneous calcium transients and contractions when pacing frequency was increased to 1 Hz. for example, in NCM 0/16 (0%) cells displayed spontaneous activity compared to 2/8 cells (25%) in HCM, and to 7/14 cells (50%) in ACM. Patch-clamp analysis of the L-type calcium current showed that compared to NCM controls, ACM treated cells had a reduced peak inward current (Ip) at 0 mV. Ip (pA/pF) was -3.31 ± 0.39 (NCM, n=7) vs -1.92 ± 0.39 (ACM, n=9); p = 0.03). Voltage-dependent channel inactivation was also changed; mid points of channel inactivation (V1/2) were -35.3 ± 2.2 mV (ACM) and -41.1 ± 1.6 mV (NCM) (p < 0.05). The ACM had no effect on voltage-dependent current activation; V1/2 was -12.98 ± 0.3 mV in NCM and -14.43 ± 0.56 mV in ACM (p =NS). These results suggest that paracrine factor(s) in the ACM predispose cardiac myocytes to calcium overload, and these factors may be important for understanding the role of adipokines in the pathophysiology of the myocardium in obesity.

**104/B51**

**Diacylglycerol Generated by Lipin Acts in Packaging Neutral Lipids into Cytoplasmic Droplets.**

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An important aspect of lipogenesis in adipose tissue is the assembly of lipid droplets, a process that is incompletely understood. Lipin, a phosphatidic acid phosphohydrolase, is known to be involved in lipogenesis through providing the substrate diacylglycerol (DAG) for triglyceride (TAG) synthesis, as well as to directly affect gene transcription of lipogenic factors. Mice with lipin mutations display a lipodystrophy-like syndrome which has certain similarities to lipodystrophy caused by mutations in seipin in humans. We show in Saccharomyces cerevisiae that the absence of PAH1 (encoding yeast lipin) results in defective neutral lipid packaging into droplets; instead, neutral lipids accumulate laterally in the ER although a few droplets remain. (Other phosphatidic acid phosphatases can provide the necessary DAG for generating TAG for filling the droplets). The droplet-packaging defect depends on the lack of phosphatidic acid phosphatase activity of Pah1p, and it is not a result of an altered triglyceride/steryl ester ratio. The packaging defect can be mimicked by overexpression of DGK1 (DAG kinase), but not by overexpression of
CDS1 (phosphatidate cytidyltransferase), indicating that the defect is caused by lack of DAG rather than a toxic effect of an excess of phosphatidic acid. This was further supported by the fact that a double knockout of both DGK1 and PAH1 restores neutral lipid packaging into droplets; this strain has the same number of droplets as wild type. We also show that Nem1p, which is known to activate Pah1p on microsomal membranes, is found in punctate structures on the ER, and most of these colocalize with seipin, which is found at ER-droplet junctions. In the absence of PAH1, much of seipin is no longer found at the ER-droplet junctions. Our data suggest that PAH1 promotes lipid droplet packaging by either introducing a shape change (via DAG) in the ER outer leaflet and/or by localizing seipin at ER-droplet junctions.

**105/B52**

**Analysis of Hormone Sensitive Lipase Activity within Adipocytes via High Content Analysis Techniques.**

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Lipid droplets are the defining feature of adipocytes and the basis for obesity, which is a worldwide health issue. Lipolysis of triglycerides occurs via lipases including Hormone Sensitive Lipase (HSL) which is phosphorylated by kinases including cAMP-dependent protein kinase (PKA, Ser 563, Ser 565, Ser 659, Ser 660), ERK-MAPK (Ser 660), glycogen synthase kinase-4 (Ser 563), Ca2+/calmodulin dependent kinase II (Ser 565) and AMP-activated kinase (Ser 565). In the basal state, HSL is found distributed throughout the cytoplasm, but HSL translocates to the lipid droplets upon activation. In this study, high content assay techniques were used to quantify HSL and lipid droplets in 3T3L1 adipocytes exposed to the β-adrenergic agonist, isoproterenol (ISO). Following treatment, the cells were fixed, labeled for nuclei, lipid droplets, & HSL (or for phosphoHSL) and then imaged with an automated digital fluorescence workstation. The images were analyzed in an automated fashion for lipid droplets and HSL on a cell by cell basis via an image analysis program which included colocalization algorithms (Pearson’s Correlation, Pr, and the Manders’ M1 coefficient). Control cells exhibited Pr values of 0.16 to 0.23, for cells visualized for HSL, signifying little association of HSL with the droplets. Exposure to 1 or 10 µM ISO led to much stronger colocalization of HSL with the droplets (Pr values of approx. 0.40). Phosphorylation of HSL on Serine 660 was dramatically increased by ISO, and the phospho-HSL Serine 660 label colocalized tightly with the lipid droplets. Z’ values > 0.6 were obtained for the effect of ISO on Pr and M1 for phospho-HSL Serine 660. The number, size, and intensity of the lipid droplets diminished upon exposure to ISO consistent with action of HSL. The high Z’ values suggest that high content analysis and phospho-HSL:lipid droplet colocalization could be used to screen chemical or genomic libraries for potential activators of HSL, furthering our understanding of the lipolysis and leading to identification of compounds with anti-obesity properties.

**106/B53**

**Mitochondrial Dysfunction and Insulin Resistance in Adipocytes.**

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Adipose tissue is an important endocrine organ which stores excess energy as triglycerides and secretes adipokines to regulate whole body energy homeostasis. We previously found that mitochondrial biogenesis occurs during adipogenesis and that impaired mitochondrial function in adipose tissue is correlated with insulin resistance in rodents and human. To study the cause-effect relationship between mitochondrial dysfunction and insulin resistance in adipose tissue, we used the 3T3-L1 adipocytes as a model. siRNA knock-down of the mitochondrial transcription factor a (Tfam), a crucial factor for mitochondrial DNA integrity and transcription, resulted in impaired mitochondrial function in 3T3-L1 adipocytes without interfering with adipogenesis. Tfam-depleted adipocytes showed no significant morphological or global gene expression profile alteration and they were normal in many aspects of adipocyte function. However, Tfam-depleted adipocytes showed impaired mitochondrial respiratory function, reflected by reduced oxygen
consumption and slower ATP synthesis rate. Insulin-stimulated GLUT4 translocation to the cell membrane and glucose transport was impaired in these adipocytes, resembling insulin resistance in adipose tissue. These results suggest that mitochondrial dysfunction could be a direct cause of insulin resistance in adipose tissue. The mechanism by which impaired mitochondrial function causes insulin resistance seems not to be a result of impaired insulin signal to AKT, since the insulin-stimulated phosphorylation of AKT in these adipocytes was, paradoxically, enhanced relative to controls. To attempt to determine the mechanism by which mitochondrial dysfunction impairs glucose transport, we analyzed gene expression profile using Affymetrix expression array. Several genes are altered in Tfam-depleted adipocytes. Among these is Rab21, a small GTPase that is localized to endocytic pathway. Preliminary data show there is a definite requirement for Rab21 in insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

107/B54
Serum- and Glucocorticoid-Inducible Kinase 1 (SGK1) Regulates Adipocyte Differentiation via Forkhead Box O1.

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Dexamethasone priming of preadipocytes is necessary for their adipogenic commitment and differentiation, but the mechanisms through which glucocorticoids stimulate adipogenesis have not been fully elucidated. We investigated the potential role of serum and glucocorticoid-inducible kinase 1 SGK1, as a mediator of glucocorticoid-initiated signals in adipocytes. Here we show that SGK1 is expressed in white adipose tissue and that its levels are induced in the conversion of preadipocytes into fat cells. Adipocyte differentiation is significantly diminished via siRNA inhibition of endogenous SGK1 expression, while ectopic expression of SGK1 in mesenchymal precursor cells promotes adipogenesis. The SGK1-mediated phenotypic effects on differentiation parallel changes in the mRNA levels for critical regulators and markers of adipogenesis, such as PPARγ, C/EBPα and fatty acid binding protein aP2. We demonstrate that SGK1 affects differentiation by direct phosphorylation of FoxO1 thereby changing its cellular localization from the nucleus to the cytosol. In addition we show that SGK1-/- cells are unable to relocalize FoxO1 to the cytosol in response to dexamethasone. Together these results show that SGK1 influences adipocyte differentiation by regulating FoxO1 phosphorylation and reveal a potentially important function for this kinase in the control of fat mass and function. We are tempted to speculate that SGK1 specific inhibitors could reduce adipogenic conversion in Cushing patients and become potentially useful therapeutic agents in the treatment of their obesity.

108/B55
Transcriptional Activation of Peroxisome Proliferator-Activated Receptor-γ during Adipocyte Differentiation.

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Peroxisome proliferator-activated receptor-γ (PPAR-γ) is widely known as a transcription factor that is important for the induction of adipogenesis. We demonstrate that transcriptional activity of PPAR-γ is regulated by both protein kinase a (PKA) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways. Transcriptional activity of PPAR-γ was markedly increased by isobutylmethylxanthine (IBMX) whereas insulin and dexamethasone was not effective. IBMX-induced activation of PPAR-γ was completely blocked by either PKA inhibitor (H89) or exchange protein activated by cAMP inhibitor (Epac, GTI-298). Furthermore, PI3K inhibitor (LY294002) completely blocked IBMX-induced transcriptional activation of PPAR-γ. Transcriptional activation of PPAR-γ was observed in the presence of both PKA agonist (6-MB-cAMP) and Epac agonist (8-pCPT-2′-O-Me-cAMP). In addition, Epac agonist strongly induced the phosphorylation of Akt. Finally, knock-down of Akt1 resulted in blunting of PPAR-γ transcriptional activation. Given these
results, we suggest here that both PI3K/Akt and PKA signaling pathways are necessary for the transcriptional activation of PPAR-γ.

109/B56
Decreased PPARγ Expression Compromises Perigonadal Specific Fat Deposition and Insulin Sensitivity.
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Mutations and polymorphisms in PPARG have been linked to adiposity and partial lipodystrophy in humans. However, how disturbances in PPARG lead to depot-specific effects on adipose tissue, as shown by the characteristic aberrant fat distribution in patients, remains unclear. By manipulating the 3'-untranslated region of the PPARγ gene, we have generated mice with PPARγ gene expression ranging from 25% to 100% normal. Basal levels of PPARγ transcripts between 50%~100% had no significant effect on body weight, fat mass, and insulin sensitivity. In contrast, mice with 25% normal PPARγ expression exhibited reduced body weight and total fat mass, insulin resistance, and dyslipidemia. Interestingly, fat mass was selectively reduced in perigonadal depot without significant changes in inguinal and other depots. Expression of adipogenic factor C/EBPα and some other metabolic genes containing peroxisome proliferator response element were reduced in a perigonadal depot-specific fashion. This was further associated with depot-specific reduction in the expression of adipokines, increased expression of TNFα, and increased ectopic lipid deposition in muscles. Together, these results underscore the differential sensitivity of the individual fat depots on PPARγ availability as an underlying mechanism of partial lipodystrophy.

110/B57
Automated Analysis of Steatosis in Hematoxylin/Eosin Stained Human Liver Biopsies.
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Excess lipid droplets (steatosis) in the liver is the defining characteristic of non-alcoholic fatty liver disease. Hepatic steatosis is also prominent in patients infected with hepatitis C virus, a disease that afflicts over 100 million people worldwide. Steatosis often progresses to fibrosis, cirrhosis, and hepatocellular carcinoma, and liver biopsies are often taken from patients to aid in diagnosis of liver dysfunction and from subjects undergoing clinical trials relevant to these disease states. Sections obtained from the biopsies are stained with hematoxylin plus eosin (H & E staining), and are commonly evaluated for steatosis by pathologists utilizing a 0, 1, 2, 3, or 4 scoring system. The goal of this study was to develop an automated algorithm for quantification of steatosis in images obtained from human liver biopsies visualized with an Aperio slide scanner. To do this, an algorithm, previously developed to quantify of lipid droplets in images obtained from cultured cells by fluorescence microscopy, was adapted for use with bright field images obtained from H & E staining procedures. After calibration with reference images, the algorithm was able to predict the steatosis score by a qualified pathologist with an accuracy of 85% (51 of 60 biopsies). A second algorithm was also developed which accurately identified nuclei within the images (the % error between automated analysis and counting of nuclei by the pathologist averaged 3% over 14 images). The development of a steatosis algorithm will enable researchers to quantify lipid droplets and predict the steatosis score from images derived from the livers of human and animal models of fatty liver and related afflictions.

111/B58
Disruptions in Sphingolipid Metabolism Induce Obesity-Like Phenotypes in Drosophila Melanogaster.
The objective of our study was to determine whether disruptions in sphingolipid metabolism affect energy homeostasis using flies as a model organism. We used a systems biology approach, utilizing lipidomics, high-throughput biochemical assays, and DNA microarrays to analyze sphingolipid (SL) mutant flies of interest. Using electrospray ionization liquid chromatography tandem mass spectrometry (ESI/LC/MS/MS), we profiled the sphingolipidome of SL mutant flies. The sphingolipidome included measurements of ceramide, sphingoid bases and their derivatives. Obesity-like phenotypes were characterized using classic hallmarks of obesity. These included increased triglyceride levels, fat body cell size, adiposity and resistance to starvation induced death. Finally, DNA microarray analysis was performed to identify potential changes in transcriptional programs related to energy homeostasis. Our results show that obesity-like phenotypes correlated with ceramide accumulating SL mutants. Furthermore, these mutants exhibit changes in their transcript expression profiles that suggest a program shift towards lipid storage and/or increased nutritional uptake. Based on these results, we conclude that SL metabolism plays an important role in regulating energy homeostasis.

Peripheral Serotonin Enhanced Bile Acids Circulation and Accelerated Lipid Metabolism.

Serotonin is a neurotransmitter synthesized in the raphe nuclei of the brain stem and involved in the central control of food intake, sleep and mood. Serotonin is also a peripheral hormone produced by enterochromaffin cells in the intestine and involved in vasoconstriction, haemostasis and immune system. Serotonin is synthesized by two distinct tryptophan hydroxylase (TPH) rate-limiting enzyme in brain (TPH2) and in peripheral (TPH1). As serotonin is inability to cross the blood-brain barrier, there are two serotonin systems in brain and periphery with independent functions. We revealed that serotonin decreased the body-weight gain of mice fed a high fat diet. However, the functions of serotonin in periphery have not yet been fully elucidated. In this study, we investigated the physiological effect of serotonin on plasma metabolites and liver functions. After fasted mice were intraperitoneally injected with 1 mg serotonin, plasma glucose level was elevated. In contrast, plasma triglyceride, cholesterol and NEFA concentrations decreased following serotonin injection. Next, we evaluated the effect of serotonin on liver functions and bile acids circulation. The hepatic glycogen content and expression of glycogen synthase 2 (GYS2) mRNA was significantly highest at 240 min after injection. at the same time, the hepatic triglyceride content was significantly lowest. Furthermore, serotonin stimulated the contraction of gallbladder and the excretion of bile, and the bile volume at 60 min was smallest. at the same time, the plasma bile acids concentration increased. In contrast, the content of fecal bile acid excretion decreased at first and second days after injection. The mRNA and protein expression of apical sodium-dependent bile acid transporter (ASBT) in ileum was significantly increased at 30 min and 240 min after injection, respectively. These data indicated that serotonin induced physiological actions in periphery via enhancing of bile acids circulation.

Plasmin/Plasminogen Facilitates Uptake of agLDL by Macrophages.

Macrophage uptake of aggregated matrix-anchored LDL leads to foam cell formation and progression of atherosclerotic lesions. Urokinase plasminogen activator receptor (uPAR) is expressed at elevated levels in atherosclerotic human arteries, primarily in macrophages. Correlations between increased plasminogen activation and accelerated atherosclerosis are also
reported in several human studies. However, a coherent picture of the role of the uPAR/plasminogen system in atherogenesis has not emerged. In order to understand the mechanisms through which the uPAR/plasminogen system accelerates atherosclerosis, we employed an In Vitro cell culture model to examine the effects of plasmin on macrophage agLDL interactions. In this system, agLDL is retained for prolonged times in surface-bound membrane invaginations termed surface-connected compartments (SCCs). Data will be presented that demonstrates plasmin treatment results in proteolysis of agLDL inducing dramatic changes in SCC morphology. Specifically, plasmin proteolysis causes surface-associated agLDL to become partially or completely internalized in an actin-dependent manner. This results in the delivery of a significant amount of cholesterol to the macrophage. Additionally, surface expression of uPAR increases during incubation of macrophages with agLDL resulting in an increased capacity to convert plasminogen into plasmin. Our preliminary data suggest a model for the mechanism of plasmin accelerated atherosclerosis in which proteolysis of agLDL causes changes in SCC morphology which facilitates cholesterol uptake by macrophages and consequent foam cell formation.

114/B61
A Novel Marker (PC-Cholesterol Complex) for Foam Cell Death Can Be Related to Oxidized Phospholipid Production.
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[Objective] Phosphatidylcholine (PC)-cholesterol (FC) complex structures, a unique atheroma related antigen related to foam cell death. To elucidate the role of antigen in the development of atherosclerosis, the relationship among PC-FC complex, FC increase and Oxidized lipid production in cultured foam cell were investigated. [Method] J774 cells incubated in the presence of hyperlipidemic sera or modified LDL for 24 hr, then cultured up to 3-13 days. PC-FC complex and Oxidized Phospholipid were detected by immunofluorescent microscopy using specific monoclonal antibodies. Cell Apoptosis was detected by Annexin V binding. [Results & Discussion] Using human or WHHL rabbit hyperlipidemic sera and J774 cells, we succeeded to express PC-FC complex as a apoptosis marker on the surface of FC-rich lipid droplets. After apoptosis of the foam cells, residual lipid droplets can be accumulated to another preparation of macrophage(2nd stage). Oxidized lipids significantly increased during the foam cell culture detected by DPPP. Oxidized phospholipids specifically accumulated in the 2nd stage foam cells whereas, no significant increase of oxidized one can be observed after treatment with U18666A in 1st stage foam cells in order to block cellular cholesterol transport. This phenomena would be related to the PC-cholesterol complex formation as well as the foam cell death. This antigen expression can be related to oxidized lipid production. Oxidized lipid accumulation might be related to the foam cell death and the expression of PC-cholesterol complex. There were significantly relationship between the PC-FC complex formation, foam cell death and oxidized lipid accumulation in 2nd stage foam cells

115/B62
Decreased Expression of N-Cadherin in Microvascular Pericytes and Endothelial Cells: Implications for Altered Vascular Permeability in Diabetic Retinopathy.
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N-cadherin is an integral membrane protein that plays an important role in microvascular function by mediating the homophilic interaction between endothelial cells and pericytes. In diabetic retinopathy, pericyte loss is considered to be an important mechanism that leads to altered blood retinal barrier function and neovascularization. Loss of N-cadherin in microvascular pericytes and endothelial cells in diabetic conditions may alter the ability of the cells to adhere with each other, resulting in pericyte loss and altered endothelial cell behavior. The objective of this study was to examine changes in the expression of N-cadherin in human microvascular endothelial cells and
pericytes exposed to high glucose (30.5 mM) that mimics the In Vivo diabetic condition. Analysis of endothelial cells and pericytes by real time PCR and western blot analysis revealed a greater than 30% reduction in the expression of N-cadherin mRNA and protein when cells were grown in high glucose compared to normal physiological glucose levels (5mM). Recently, Angiopoietin-2 (Ang-2) has been shown to be up regulated in cells in response to high glucose treatment and increased Ang-2 induces pericyte loss in animal studies. Our studies have revealed that treatment of both pericytes and endothelial cells with Ang-2 for a minimum of 48 hours leads to reduced expression of N-cadherin. In addition, rats receiving an intraocular injection of Ang-2 revealed a significant (26%) decrease in the level of N-cadherin 24 hours following the injection. Further studies utilizing immunocytochemical analyses demonstrated that the expression of N-cadherin by endothelial cells is regulated in part by the presence of pericytes in the local environment. Results from these studies suggest that high glucose and increased levels of Ang-2 in the diabetic condition could lead to altered N-cadherin expression in microvascular cells. Reduced N-cadherin may further lead to modification of endothelial cell/pericyte interactions and alteration of the blood-retinal-barrier.

116/B63
Improvement of Mouse Embryo Progenitor-Derived Insulin-Producing Cells for Type 1 Diabetes Therapy.
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Generation of functional insulin-producing cells from stems cells is a promising approach for cure of type 1 diabetes. We have been able to differentiate mouse embryonic stem and progenitor cells into highly pure and expansible insulin-producing cells which could reverse hyperglycemia in diabetic animals. However, these cells revealed the features of immaturity and unphysiological insulin secretory response to glucose stimulation. It is known that some transcription factors act as master regulators for expression of the genes key to various aspects of islet β-cell function. One such transcription factor, v-maf musculoaponeurotic fibrosarcoma oncogene homolog a (MafA), plays important roles in promoting differentiation of mature β-cells and regulating insulin expression for maintaining β-cell phenotype. This work aimed to examine potential benefits after overexpression of MafA in our mouse embryo progenitor-derived insulin-producing (MEPI)-1 cells since MafA expression in these cells was much lower than that in isolated islets. By using a lentivirus expression system, MafA could be up-regulated by 2-fold in MEPI-1 cells after 72-h transfection. MafA-overexpressed cells exhibited better signal transduction responses to glucose stimulation as reflected by the higher glucose metabolic rate and enhancement of both membrane potential depolarization and intracellular Ca2+ concentration rises upon high glucose challenge. More importantly, MafA up-regulation markedly improved glucose-stimulated insulin secretion (GSIS) profile by suppressing the basal release and increasing the high glucose effect, displaying a dose-response curve similar to that observed in isolated islets. Meanwhile, the insulin content was increased. Real-time PCR found that the lower expression of GLP-1R, glucose transporter 2, glucokinase, NK6 transcription factor-related locus 1 and Kir6.2 in MEPI-1 cells in comparison with primary β-cells, was significantly up-regulated upon MafA overexpression. Taken together, our data demonstrated that normalization of MafA levels could enhance the β-cell relevant gene expression profile in MEPI-1 cells toward promoting maturation and substantially improve GSIS by acting on multiple signaling cascade events.

117/B64
Effect of Administration of Ethanol Extracts of Bidens Odorata, Eryngium Carlinae and Loeselia Mexicana on Diabetic Rats.
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Diabetes mellitus is a metabolic disorder of multiple etiologies, characterized by chronic hyperglycemia. This chronic hyperglycemia is directly related to the development of long-term
complications, diabetic nephropathy, among others, which determine the quality of life of diabetic patients. Because of this, there is the need to investigate new alternatives to treat diabetes based on plants known since ancient times. The aim of this study was to investigate the effect of administration of the ethanol extracts of Bidens odorata (BO), Eryngium carliniae (EC) and Loeselia mexicana (LM), plants of the forest area of the indigenous community of San Juan Nuevo Parangaricutiro, Michoacán, México in diabetic rats. We measured glucose levels in blood and weight of the rats, likewise, was measured the food and water intake for 30 days, during which conducted oral administration (in drinking water) of the three extracts in a dose of 150 mg/kg. Diabetes was induced by a single injection (ip) of streptozotocin (40 mg/kg). Rats with glucose levels above 300 mg/L were used to shape the diabetic groups. The administration of extracts of BO, EC and LM had no hypoglycemic effect; however, its use was able to reduce the weight loss characteristic of diabetic model. The symptoms of polyphagia and polydipsia were present in the diabetic groups, as evidenced by the high consumption of food and water, compared with a no diabetic group (p<0.01) despite receiving treatment with different extracts. Although, the results of the evaluated parameters did not show a clear effect of extracts of the diabetic condition, believe that their consumption is beneficial, since extracts help to overcome the suffering, the end of treatment the rats in diabetic group who received no treatment died, unlike those who did receive any of the extracts which have survived several weeks after treatment. Due to the results, currently being tested by varying the route of administration and dose of BO and EC extracts to find effective dose is also possible to evaluate the antioxidant activity of extracts in different organs. Acknowledgements: The authors appreciate the partial economic support of CIC-UMSNH (2.16, 2009) grant.

118/B65
A Novel Role for the Centrosomal Protein, Pericentrin, in Regulation of Insulin Granule Docking.

The centrosome is important for microtubule organization and cell cycle progression in animal cells. Recently, mutations in the centrosomal protein, pericentrin, have been linked to human microcephalic osteodysplastic primordial dwarfism (MOPD II), a rare genetic disease characterized by severe growth retardation and early onset of type 2 diabetes among other clinical manifestations. Case reports of children with MOPD II describe abnormalities in glucose homeostasis, yet the mechanism linking pericentrin mutations with dysregulated glucose homeostasis and diabetes is unknown. We have observed abundant expression of pericentrin in pancreatic β-cells of normal animals which led us to hypothesize that pericentrin may have a critical function during insulin secretion. Here we report an essential role for the integral centrosomal protein, pericentrin, in docking of vesicles in professional secretory pancreatic islets and insulinoma cells. In addition to the typical centrosome localization, pericentrin was also enriched with secretory vesicles in the cytoplasm. Pericentrin co-localized and co-immunoprecipitated with syntaxin-1, a core component of the SNARE complex that regulates vesicle trafficking and tethering to the plasma membrane. Pericentrin overexpression in β-cells resulted in aggregation of insulin-containing secretory vesicles with cytoplasmic pericentriolar material and an increase in the levels of intracellular insulin. RNA mediated silencing of pericentrin in secretory β-cells caused dysregulated secretory vesicle docking at the plasma membrane and hypersecretion of insulin into the media. Mice transplanted with pericentrin-depleted islets had abnormal fasting hypoglycemia. This previously unrecognized function for centrosomal protein in protein secretion illustrates the utility of these scaffolding proteins to regulate diverse cellular processes and identifies a novel target for modulating protein secretion in disorders such as diabetes.

119/B66
Identification and Characterization of a Proinsulin Interacting Protein, TMP21/P23.
Insulin is synthesized in the Endoplasmic Reticulum (ER) of pancreatic beta cells as proinsulin, transported through the secretory pathway, processed and stored in granules along with other proteins until stimulated for secretion. In order to identify proteins that interact and facilitate transport of proinsulin in the early secretory pathway, a yeast two hybrid screen was performed against a mouse pancreatic islet cDNA library. We identified and focused our studies on TMP21, or p23, a type I transmembrane protein of the p24 family of proteins that play an important but not well understood function in vesicular trafficking between the ER and Golgi. Our immunofluorescence analyses of mouse and rat insulinoma cell lines, MIN6 and INS-1 cells respectively, demonstrated that TMP21 co-localizes with proinsulin, suggesting a potential initial site of interaction. Over-expression and coimmunoprecipitation studies of various deletion mutants of TMP21 with proinsulin in COS-1 cells suggested that the luminal GOLD domain of TMP21 is sufficient to mediate this interaction. In order to analyze the functional significance of this interaction we made stable cells of MIN6 cells that had reduced expression of TMP21 as well as other isoforms of TMP21. These cells had slightly fragmented Golgi as had been seen before for cells with low levels of TMP21, but had marginal effect on the processing of proinsulin to insulin. We are currently exploring the possible function of TMP21 in post-Golgi insulin granule biogenesis, quality control in secretory granules and/or regulated insulin secretion. Our results for the first time add proinsulin to the list of other proteins such as Golgi matrix proteins (GRASP55 and GRASP65) that interact with TMP21.

120/B67
The Effect of Angelica Sinesis on Improving Renal Functions and Increasing BMP-7 Expression in Diabetic Nephropathy.
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Hyperglycemia induced reactive oxygen species (ROS) generation is believed as major factors leading to diabetic nephropathy (DN). Angelica sinensis (AS) is mentioned to show renal protective effect in combination with other herbs. Bone morphogenetic proteins-7 (BMP-7) is also produced merit in protection of DN. The role of BMP-7 in AS-induced renal improvement is not clear. The present study investigated the effects of AS on renal functions, BMP-7 expression, and the levels of ROS in streptozotocin (STZ)-induced diabetic rats and high glucose exposed rat mesangial cells (RMCs). After one- or four-week treatment, AS improved renal functions and increased renal BMP-7 expression in diabetic rats. The BMP-7 expression in RMCs was reduced by high glucose treatment and this could be reversed by AS. Moreover, RMCs exposed to high glucose were expired by BMP-7 RNAi transfection but those cells remained alive by scramble transfection. Thus, we employed regular RMCs to knock down BMP-7 with RNAi and we found that AS increased BMP-7 expression in these RMCs. Direct activation of BMP-7 expression by AS could be considered. The results of DPPH assay, DHE stain, and lucigenin assay indicated that AS could inhibit high glucose-induced ROS in RMCs. These results suggest that AS has an ability to improve renal functions in STZ-diabetic rats through increasing endogenous BMP-7 expression and decreasing oxidative stress in kidney. The present study suggests that AS could be applied to improve renal functions in diabetic disorders.

121/B68
A Functional Screen Using High Content Analysis Reveals miRNA’s That Are Involved in Regulating the Intracellular Lipid Content of Hepatocytes.
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Objective: Studies have demonstrated that 75% of obese individuals develop hepatic steatosis independent of alcohol abuse, a condition known as Non-Alcoholic Fatty Liver Disease (NAFLD).
NAFLD is progressive and can eventually lead to liver failure. Microarray studies have demonstrated more than 100 microRNA’s (miRNA’s) are misregulated in NAFLD. However, a causative link between miRNA misregulation and development of NAFLD has not been shown. Therefore, this study was undertaken to identify miRNA’s that have the ability to regulate intracellular lipid content in hepatocytes and may be involved in the development of NAFLD. Methods: Huh-7 cells, which were derived from a human hepatoma, are spontaneously steatotic under normal cell culture conditions, demonstrating several fold higher levels of triglycerides and cholesterol, when compared to normal immortalized human hepatocytes. Increased de novo lipogenesis in HUH-7 cells mimics one of the underlying causes of NAFLD, making them an ideal model for this study. Huh-7’s, were transfected with a library of 327 human miRNA’s, in duplicate 384 well plates and incubated under normal cell culture conditions for 4 days. Following incubation, the cells were fixed and stained for nuclei and lipid droplets. The well plates were then imaged using an automated microscopy platform and analyzed using automated image analysis software. for the primary screen, hits were defined as those miRNA’s which altered intracellular lipid content by +/- 30%. Hits from the primary screen were included in a secondary screen and compared to a scrambled miRNA control. miRNA’s that altered lipid content +/- 40% from the control were considered hits. Results: The screen revealed 11 miRNA’s that increased or decreased intracellular lipid content by 40% or more. The effective miRNA’s were able to increase lipid content up to 170% or decrease lipid content to 48% of control levels. Conclusions: This study demonstrates a causative link between alterations in the expression levels of certain miRNA’s and their effects on hepatocyte lipid content. Follow up studies are underway to determine the mechanism of action underlying the effects of our identified miRNA’s.

**122/B69**

**Chondrogenic Potential of Mesangial Cells in Diabetic Nephropathy.**

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Objective—Phenotypic changes of mesangial cells (MCs) are key pathologic findings during the process of progressive diabetic nephropathy (DN). MCs showed activated smooth muscle and acquired fibroblast phenotypes in addition to increased basement membrane components. This study investigated whether MCs can differentiate into chondrocytes in developing diabetic glomerular injury. Methods and Results—DN mice exhibited the induction of both SOX9, a master transcriptional factor of chondrogenesis and its downstream target, type II collagen (COL2). Positivity of COL2 was detected in sclerotic lesions in diabetic renal glomeruli shown by immunohistochemistry. So we explored this mechanism in vitro. A glomerular mesangial cell line was established from glomeruli isolated from normal, 4 week-old mice (C57BL/6JxSJL/J). Advanced glycation end-products stimulation induces the expression of BMP-2 and BMP-4 (BMP-2/4) in MCs. When cultured under micromass culture method in the presence of BMP-2/4 which triggered chondrogenesis of pluripotent C3H10T1/2 stem cells, MCs formed well-defined pellets comprising cells embedded in an extracellular matrix (ECM). In the ECM, highly expressed sulfated proteoglycans and COL2 were confirmed by Alcian blue (AB) and immunocytochemistry. The expression of SOX9 and COL2 was upregulated by BMP-2/4 in western blotting. SOX9 was also upregulated by hypoxic condition. COL2 was suppressed by treatment with a p38 kinase inhibitor, SB203580. Overexpression of SOX9 caused ectopic expression of sulfated proteoglycans and COL2 proven by RT-PCR, and western blot analysis, AB and immunocytochemistry. Conclusions—our present data showed ectopic BMP-2/4 expressions promote differentiation of MCs into chondrocytes by induction of SOX9 expression. It provide a new aspects in the progression of glomerular injury—MCs have a chondrogenic potential. As chondrocytes are unique cells that locate at avascular and hypoxic condition, it is implied that the transdifferentiation of MCs into chondrocytes is an accommodation response to chronic pathological hypoxia in kidney. And our data also suggest under pathological states, impaired differentiation of MCs may contribute to the progression of DN.
Glucose-6-phosphate dehydrogenase (G6PD), one of the NADPH producing enzymes, plays crucial roles in lipid metabolism and cellular redox regulation. Recently, we have reported that overexpression of G6PD in adipocytes stimulates lipogenesis and insulin resistance via increase in reactive oxidative species (ROS) generation and inflammatory response (Mol. Cell. Biol. 25:5146-5157, 2005; Diabetes 55:2939-2949, 2006). The fact that chronic oxidative stress causes pancreatic b-cell failure prompts us to elucidate whether alteration of G6PD is also linked with pancreatic b-cell dysfunction. In this study, we observed that G6PD expression was greatly increased in the pancreatic islets of several diabetic animal models including db/db mice and OLETF rats. In addition, G6PD expression was increased by FFAs and TNFα, which are mediated in metabolic syndromes. Furthermore, SREBP-1c, a well known transcription factor for lipogenesis and b-cell dysregulator, directly regulated expression of G6PD. When G6PD was overexpressed in INS-1 insulinoma cells, b-cell specific gene expression including PDX-1, insulin, and Glut2 was significantly down-regulated. Furthermore, G6PD overexpressing INS-1 cells exhibited increased expression of pro-oxidant enzymes with a little change of expression of antioxidant enzymes, resulting in ROS accumulation. Subsequently, enhanced G6PD expression led to apoptosis of pancreatic b-cells, concomitant with increases of pro-apoptotic gene expression as well as NF-kB activation. Taken together, these findings suggest that increased G6PD expression in pancreatic b-cells would elevate cellular ROS accumulation and activate NF-kB signaling, resulting in a b-cell dysregulation, which is frequently found in type 2 diabetic subjects.

Increased Fat Due to Estrogen Deficiency Induces Bone Loss by Elevating MCP-1 Production.

Growing evidence links fat and bone metabolism. Increased adipose tissue mass is associated with changes in the endocrine and metabolic function of adipose tissue. Altered production of adipokines by adipose tissue has been implicated in metabolic consequences associated with obesity. Adipose tissue of obese mice expressed increased level of monocyte chemoattractant protein-1 (MCP-1, CCL2), compared with that lean mice (Sartipy and Loskutoff, 2003). Ovarian involution and estrogen deficiency induce postmenopausal bone loss through effects on bone marrow and bone cells. Steady-state bone mass depends upon a balance between rates of bone formation and bone resorption. The most relevant consequence of estrogen deficiency due to ovariectomy (OVX) is elevated cytokine-induced osteoclast (OC) formation (Weizmann and Pacifici, 2006). We have investigated the relationship between the increased fat and the bone loss associated with OVX-induced estrogen withdrawal. We have focused on the effect of fat has on OC formation by releasing MCP-1. This work was supported by a KHIDI Grant (A090080), a KRF Grant (KRF-2008-314-C00249), a KOSEF grant (R012007000210820), and a KRF Grant (2009-0066232) funded by the Korean government.

Ciliary Transition Zone Proteins Are Required for Proper Basal Body Positioning, Structural Integrity of the Ciliary Gate, and Proper Formation of the Axoneme.
Cilia are organelles with motile and/or sensory-signaling functions that protrude from most metazoan cell types and are important for a wide array of physiological and developmental processes. The most proximal section of the ciliary axoneme, which adjoins the basal body (the organelle required for ciliary axoneme elongation), is termed ‘transition zone’. Approximately 12 different proteins localise within this region, most of which are implicated in so-called ‘ciliopathies’ (e.g., Meckel syndrome, nephronophthisis, Joubert syndrome) that are characterised by retinal degeneration, kidney disease, brain and skeletal malformations, and other ailments. Remarkably, the function of these transition zone-associated proteins remains enigmatic. Here we use C. elegans to demonstrate functional interactions between many of these proteins, and show that they are required for several aspects of ciliogenesis. Various double mutant combinations, analysed by both fluorescence microscopy of ciliary markers and transmission electron microscopy, reveal defects in basal body positioning, formation of the ciliary gate formed by transitional fibers and Y-links at the base of cilia, and axoneme formation. Our findings expand the interaction network of ciliary proteins, describe previously unknown ciliogenic roles for the transition zone region, and point to the importance of ciliopathy-associated proteins in early ciliogenic processes.

126/B73
Restriction of Access into the Ciliary Membrane Requires MKS and NPHP Proteins.
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Cilia are microtubule-based and membrane-bound organelles that serve diverse functions in various organisms and tissues. The ciliary membrane is a tightly regulated region that is enriched with many proteins not found throughout the rest of the cell membrane. Likewise, many proteins found in the cell membrane are excluded from the cilium. The mechanisms by which the cell membrane and ciliary membrane are differentially regulated are unknown. It is proposed that proteins at the base of the cilium participate in cell/ciliary membrane protein sorting, but the molecular identities of such proteins have not been uncovered. Here, we demonstrate that cilia Basal body proteins implicated in the human developmental disorders Nephronophthisis (NPHP) and Meckel-Gruber Syndrome (MKS) function in this capacity in Caenorhabditis elegans. Using a combination of gene mutations and fluorescence-tagged transgenes, we find that NPHP-1, NPHP-4, MKS-1, MKSR-1, MKSR-2, MKS-3, and MKS-6 function redundantly in the worm to maintain proper cilia morphology such that mutations in single genes have little or no effect on ciliogenesis while certain combinations of mutant genes are detrimental to cilia formation. Although singularly not required to build cilia in C. elegans, these genes influence cilia sensory signal transduction. We propose that at least part of this influence is through maintaining the compositional integrity of the ciliary membrane. We find that in the absence of NPHP-1, NPHP-4, MKSR-1, MKSR-2, or MKS-6, transmembrane and membrane-associated proteins normally excluded from the ciliary membrane are allowed to freely access or accumulate within the cilium. Such proteins we observed abnormally entering the cilium include TRAM-1a, RP2, and MKS-3. These results contrast previous studies in which disruption of other cilia-associated disease proteins resulted in the loss of some normally cilia-localized proteins from the cilium. These data provide important insight into a mechanism by which ciliary membrane composition and signal transduction is regulated and may underlie the molecular basis of phenotypes observed in NPHP and MKS patients.

127/B74
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In Polycystic Liver Disease (PCLD), benign hyperproliferation of cystic cholangiocytes and cell cycle dysregulation contribute to hepatic cystogenesis. We showed recently that dysregulation of cholangiocyte cycle in PCK rats (animal model of PCLD) results from Cdc25A overexpression due to decreased miR-15a levels. In many cancers, abnormal cell cycle is associated with p53-dependent centrosome hyperamplification. However, no data exist on centrosome hyperamplification and mechanisms involved in this process in benign proliferative conditions such as PCLDs. Our aims were to analyze: (i) centrosome morphology and numbers; (ii) expression of p53 and its targets: p21, Cdk2/CyclinE; (iii) role of Cdc25A in centrosome hyperamplification; and (iv) effect of Cdc25A modulation by miR-15a on centrosome amplification in PCK-CCL (cholangiocyte cell line derived from PCK rats). Centrosome morphology and numbers were assessed by confocal or transmission electron microscopy In Vivo (normal and PCK rat livers) and In Vitro [PCK-CCL and normal rat cholangiocytes (NRC)]; expression of proteins by Western blot; and cell cycle by flow cytometry. PCK-CCL was transfected with a miR-15a precursor to decrease Cdc25A level and analyze effect of this manipulation on centrosome numbers. 30% of PCK-CCL had amplified centrosomes compared to 6% in NRC. Majority of PCK-CCL were in G2/M phase in contrast to NRC (mostly present in G0/G1 phase). Centrosomes in cystic cholangiocytes were longer, improperly positioned, with additional appendages; cells with multiple spindles were observed. Levels of p53 and p21 were not different between PCK-CCL and NRC and no mutations were found in p53 gene. However, Cdk2 and Cyclin E levels were ~2-times higher, likely due to Cdc25A over-expression (by 75%). Transfection of PCK-CCL with a miR-15a precursor inhibited Cdc25A up to 50% and decreased the number of cells with hyperamplified centrosomes by 10% (p<0.05). In the PCK-CCL, centrosome hyperamplification is linked to Cdc25A overexpression. Experimental suppression of Cdc25A by miR-15a reduces the number of cells with hyperamplified centrosomes. The data suggest that centrosome hyperamplification in PCLD appears to be Cdc25A but not p53-dependent.

128/B75
Activation of Trpv4 Reduces the Hyperproliferative Phenotype of Cystic Cholangiocytes from an Animal Model of ARPKD.
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In the polycystic liver diseases, formation of hepatic cysts involves benign hyperproliferation of cholangiocytes. In PCK rats, an animal model of autosomal recessive polycystic kidney disease (ARPKD), cholangiocytes have decreased [Ca^{2+}]i levels that contribute to cholangiocyte hyperproliferation. We recently showed that Trpv4, a calcium entry channel, is expressed in normal rat cholangiocytes and its activation leads to an increase in [Ca^{2+}]i. Thus, we hypothesized that pharmacological activation of Trpv4 might reverse the hyperproliferative phenotype of PCK cholangiocytes. Methods: Trpv4 expression was examined in liver tissue from normal and PCK rats and from normal humans and patients with both autosomal dominant polycystic kidney disease (ADPKD) and ARPKD. Trpv4 expression was examined in liver tissue from normal and PCK rats and from normal humans and patients with both autosomal dominant polycystic kidney disease (ADPKD) and ARPKD. The effect of Trpv4 activation on PCK cholangiocyte proliferation and cyst formation was assessed in cultured cholangiocytes derived from normal and PCK rats. Results: We found Trpv4 was overexpressed ~8 times at the mRNA level and ~3 times at the protein level in PCK cholangiocytes. Confocal and immunogold electron-microscopy supported Trpv4 overexpression in cholangiocytes of PCK rats and human samples of patients with ARPKD or ADPKD. Treatment of PCK cholangiocytes with Trpv4 agonists (4oPDD, 5',6'-EET and nifedipine+arachidonic acid) increased [Ca^{2+}]i levels by ~30% resulting in inhibition of cell proliferation by ~25%-50% and cysts growth in 3-D-culture by ~70%. The inhibitory effects of Trpv4 activators on cyst growth were blocked when Trpv4 was silenced with a specific siRNA. Attenuation of cell proliferation and cyst growth in response to the increase in [Ca^{2+}]i was associated with activation of Akt and inhibition of β-Raf and Erk1/2. Conclusions: Cholangiocytes from PCK rats and humans with ARPKD and ADPKD overexpressed the calcium channel, Trpv4. Pharmacologic activation of Trpv4 by three different agonists increases levels of [Ca^{2+}]i in cystic cholangiocytes from PCK rats, leading to inhibition of cholangiocyte hyperproliferation and In Vitro
cyst growth via a mechanism involving Akt activation and inhibition of β-Raf/Erk pathway. These data suggest that TRPV4 may represent a potential therapeutic target in the polycystic liver diseases.

129/B76
Novel Cellular Distribution of Foxj1.
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Objective: to confirm Foxj1 as a specific marker of ciliated epithelial cells, we evaluated the cellular localization of Foxj1 in the mouse lung. Method: NBF fixed tissues were embedded in paraffin & sectioned to 5-µm thickness. Sections were deparaffinized then antigen retrieval was performed. Sections were blocked in 10% serum, rabbit α-Foxj1, goat α-CC10 & mouse α-β tubulin IV were incubated with sections for 1h (IHC) & O/N (immunofluorescence or IFA) then in IHC to visualize the signal anti-rabbit & anti-goat ABC kits were used along with Nova red substrate. For IFA after primary antibody Alexa 488 donkey anti-rabbit or Cy3 anti-goat or Alexa 488 anti-mouse were used. Stained sections were imaged using Nikon fluorescence microscope. For western blot lung lysate was prepared by homogenizing the whole lung then cytoplasmic & nuclear fractions were separated using BioVision extraction kit. Samples were run on 10% SDS-PAGE and transferred on to PVDF membrane. The membrane was incubated with odyssey blocker for 1 hr then with anti-Foxj1 antibody & anti-CC10 antibody for O/N at 4°C. These membranes were incubated with IRDye 800-anti-rabbit & IRDye-800 anti-goat antibody then imaged using Li-Cor Odyssey imaging system. Summary: Foxj1 is a transcription factor, involved in the regulation of ciliogenesis & expressed in the nucleus of ciliated cells of various tissues including respiratory tract. The distribution of Foxj1 in FFPE lung tissues using a harsh antigen retrieval method was nuclear as previously reported, but we found its also cytoplasmic & associated with the cilia. These findings were confirmed using double immunofluorescence which demonstrated colocalization of Foxj-1 & β-tubulin IV in the cilia & cytoplasm of respiratory ciliated epithelial cells. CC10, a marker of Clara cells & Foxj1 were mutually exclusive in their expression by IFA. Western blots of the cytosolic & nuclear fractions of mouse lung identified two forms of Foxj1: a ~45 KD almost exclusively nuclear form, and ~50 KD nuclear and cytoplasmic form. Conclusion: Foxj1 can be used to identify specifically ciliated cells in the mouse lung. A novel pattern of sub-cellular distribution of two forms of Foxj1 in the mouse lung was established.

130/B77
Follicle-Stimulating Hormone (FSH) Stimulates Biliary Growth in the Course of Autosomal Dominant Polycystic Kidney Disease.
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Cholangiocytes, the epithelia lining the biliary epithelium, are the target cells of several human liver diseases defined as cholangiopathies. Autosomal dominant polycystic kidney disease (ADPKD) represents an important example of a genetic cholangiopathy, since it is caused by mutations in PKD genes with an abnormal formation of multiple renal and biliary cysts. Biliary hyperplasia is regulated by a number of factors including sex hormones such as estrogens, progesterone and follicle-stimulating hormone (FSH). We have previously shown that FSH stimulates cholangiocyte growth by activation of cAMP signaling. Here, we tested the hypothesis that FSH regulates the growth of ADPKD biliary cysts by an autocrine mechanism. Methods: We evaluated: (i) the expression of FSH and FSH receptor (FSHR) by immunofluorescence and real-
time PCR; and (ii) cholangiocyte proliferation in a. liver sections from normal and ADPKD patients (by immunohistochemistry for PCNA) and b. immortalized normal human cholangiocyte lines (H69) and immortalized cell lines obtained from the epithelium lining the hepatic cysts from patients with ADPKD (LCDE) by immunoblots for PCNA. In H69 and LCDE cells we assessed FSH secretion in short-term cultures (6 hours) by EIA kits and intracellular cAMP levels by RIA kits. Also, we used siRNA to knockdown FSH expression in LCDE and H69 cells before evaluating cAMP levels and proliferation by PCNA immunoblots. Results: There was: (i) a higher expression of FSH (~60%) and FSHR (~30%); and (ii) elevated cholangiocyte proliferation in sections from ADPKD patients compared to normal patients and in LCDE cells compared to normal H69 cells. There was increased FSH secretion (~40%) in cultures of LCDE compared to H69 cells. Silencing of FSH induced a decrease in cAMP levels and cholangiocyte proliferation in ADPKD samples and LCDE cells. Conclusion: FSH is an important autocrine trophic factor for biliary growth in the course of ADPKD. Modulation of cholangiocyte FSH secretion may be important in the control of the formation and the progression of hepatic cystogenesis.

**131/B78**

**The NPHP Cystic Suppressor Proteins Form a Centriolar Compartment Required for Formation of the Primary Cilium.**

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Nephronophthisis (NPHP) is an autosomal recessive, cystic kidney disease, caused by mutations of nine known genes. NPHP patients also show cerebellar defects, situs inversus, and retinitis pigmentosa. The molecular mechanism suppressing formation of cysts is poorly understood, but may include changes in the apical-basal polarity of cells, cell cycle, or secretory behavior of cells. Recent studies suggest that specific changes in ciliary signaling mechanisms may be important for cyst suppression. Using a recently reported system for high-throughput protein tagging and proteomic analysis (PMID: 19405035), we have generated inner medullary collecting duct (IMCD) and fibroblast cell lines stably expressing GFP (LAP)-tagged forms of NPHP1, NPHP2/Inversin, NPHP3, NPHP4, NPHP5, NPHP6/CEP290, and NPHP8/RPGRIP1L. We have identified a network of high confidence interaction proteins placing the NPHP proteins in three subnetworks. In the first network, strong interactions among NPHP1, NPHP4, and NPHP8 are linked to a cortical network of actin binding proteins and show apical localization in IMCD3 cells, but are not strongly required for ciliation. In the second network, NPHP6 binds to and directs NPHP5 proteins to the centriole. NPHP5 also binds NPHP2, which localizes to both centriole and cilium. NPHP5 is organized at the cortex by association with the exocyst proteins Sec3/Sec8, calmodulin, and NPHP1. NPHP5 binds to the deubiquitinating enzyme USP9X/Fat Facets. Both NPHP5 and USP9X are required for ciliation in RPE cells, suggesting ubiquitin-dependent control of ciliation. In the third network, NPHP6/CEP290 associates with MKS1 and MKS6, proteins mutated in the related Meckel-Gruber syndrome. MKS proteins participate in pathways important for neural tube closure and Hedgehog signaling. We did not observe direct interactions among the NPHP subnetworks and proteins associated with intraflagellar transport complexes IFT-A or IFT-B, nor proteins important in the Bardet-Biedl syndrome (BBS) or ciliopathy. We suggest that NPHP proteins participate in a centriolar-cortical anchoring mechanism.

**132/B79**

**Planar Cell Polarity (PCP) Proteins Regulate a Novel Apical Localization of Polycystin-1.**

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Autosomal dominant polycystic kidney disease (ADPKD), which leads to forming cyst instead of developing proper renal tubules, is one of most common inherited diseases of humans. at least 85% of all cases of ADPKD result from mutation of the PKD1 gene, encoding the protein polycystin-1 (PC-1). However, the mechanisms by which PC-1 regulates the diameter of renal tubules and the reasons why ADPKD patients with mutations of PKD1 develop kidney cysts remain obscure. Recent studies suggest that cysts may result from improper establishment or maintenance of planar cell polarity (PCP). As a first step in determining the role of PC-1 in this
process, we examined the cellular localization of PC-1 with respect to a set of known PCP proteins. Because endogenous levels of PC-1 are too low to detect with certainty, we developed an MDCK cell culture system with inducible expression of EGFP-tagged PC-1. Using this system, we identified a previously undescribed apical pool of PC-1 that co-localizes with Dishevelled (Dvl) and Vangl2. Interestingly, the apical localization of PC-1 appears to be inhibited by over-expressing dominant negative Dvl or knocking down Vangl2, suggesting that Dvl and Vangl2 may play a role in regulating PC-1 sub-cellular localization. This localization appears to be essential for proper tubule formation since a PC-1 mutant that does not localize apically is also defective in forming tubules in vitro. Finally, we investigated PC-1 signaling downstream of PC-1 and found enhanced RhoA activation, localized to the apical membrane near PC-1.

133/B80  
**Aurora-A Kinase Regulates the PKD2 Calcium Channel.**

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Autosomal dominant polycystic kidney disease (ADPKD) is a common condition, affecting 1 in 800 live births from all ethnic groups. The majority of the cases arise from inherited mutations in the PKD1 and PKD2 genes, which encode the proteins polycystin 1 and 2 (PC1 and PC2). PC2 belongs to the transient receptor potential (TRP) superfamily of transmembrane ion channels, and functions at the endoplasmic reticulum (ER) as a Ca2+ release channel. PC2 also heterodimerizes with PC1 at the membrane of the primary cilium, where it acts as mechanosensitive channel. Downregulation of PC2 calcium channel activity is associated with PKD pathogenesis. Aurora a (AurA) is a centrosomal kinase that is best known for a role regulating mitotic entry and promotes progression through the stages of mitosis. We have now shown that AurA directly binds the C-terminal cytoplasmic domain of PC2 and phosphorylates a unique residue an S829 residue. AurA phosphorylation negatively regulates PC2 channel activity. Small molecule inhibition of AurA phosphorylation or depletion of AurA by specific siRNA increases Ca2+ release through the PC2 channel, while AurA overexpression decreases Ca2+ release. Physiological stimuli that normally transiently increase PC2 activation and intracellular calcium level also rapidly and transiently induce AurA activation, with a time course appropriate for AurA limitation of PC2 activity. Our data suggest that AurA is activated by the cytoplasmic Ca2+ released from ER stores, then binds and phosphorylates PC2 to suppress further PC2 channel activity. AurA is abundantly expressed in human and murine kidneys and activated phAurA is present in the kidneys cells lining ADPKD cysts. Our work provides the first evidence that AurA acts as a rapid response environmental sensor in mammalian cells. We conclude that AurA is novel regulator of PC2 activity, and hypothesize that AurA inhibition may have therapeutic benefit in ADPKD treatment.

134/B81  
**Purinergic Signaling Can Regulate the Cell Surface Delivery of Polycystin-1.**

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Polycystin-1 (PC1) is a mechano- or chemosensory receptor that localizes to the plasma membranes and cilia of epithelial cells. Mis-localization of PC1 may be associated with cystic kidney disease, and a variety of partner proteins and intracellular conditions are likely to regulate PC1 expression and delivery to the plasma membrane. The ion channel polycystin-2 (PC2) and PC1 interact with one another through their C-terminal tails. PC2 is known to modify the localization of PC1 in cultured cells, and PC1 may affect PC2 channel function. Using a quantitative surface immunofluorescence assay, we found that PC1 requires co-expression with PC2 to reach the plasma membrane in cultured HEK293 cells. PC1 surface localization requires functional PC2 channel activity, as demonstrated by the failure of a channel-defective PC2 mutant to support surface delivery. Interestingly, surface delivery does not require a stable
interaction between PC1 and PC2, since C-terminally truncated versions of either protein do not negatively affect PC1 localization. The delivery of PC1 to the surface is correlated with an increase in the cleavage of PC1 at the G-protein coupled receptor proteolytic site (GPS), a cleavage that is necessary but not sufficient for surface delivery. We find that extracellular ATP acting through cell surface purinergic receptors potentiates the effect of PC2 on PC1 localization. Adding extracellular ATP increases the amount of PC1 that reaches the surface in the presence of PC2, while the purinergic receptor blocker suramin abrogates the effect and reduces the amount of surface PC1, even in the presence of PC2. ATP seems to affect steps late in the secretory pathway, since the ATP-induced increase in surface PC1 is not accompanied by an increase in the GPS cleavage. In summary, our data indicate that PC1 localization to the plasma membrane depends on the channel activity of PC2 and that extracellular ATP, acting through purinergic receptors, may regulate this delivery.

135/B82
Ciliary Membrane Protein Trafficking in C. elegans.
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Cilia are sensory organelles that protrude like antennae off eukaryotic cells. Receptors and signaling transduction molecules dynamically localize to the cilium in a tightly regulated process. Defects in ciliary development, formation, or function may result in human genetic diseases, or ciliopathies. We are using the nematode Caenorhabditis elegans to study ciliary receptor trafficking [1] and also to model human ciliopathies. A group of ciliopathies including Meckel-Gruber Syndrome (MKS), Joubert’s syndrome, and nephronophthisis (NPHP) display overlapping clinical manifestations and causal genes. The C. elegans genome encodes homologs for several of the MKS, Joubert’s, and NPHP genes, and these gene products specifically localize to the cilia base. We hypothesize that these proteins act at the ciliary base and function in membrane protein localization. PKD2 (Polycystic Kidney Disease gene 2) encodes a conserved TRP polycystin (TRPP) channel that localizes to cilia and may act as a mechanosensor. We have been looking at the role of the MKS, Joubert’s, and NPHP genes on the localization of PKD-2 and other ciliary components in C. elegans. Mammalian INPP5E mutations cause Joubert’s syndrome [2, 3], whereas, the C. elegans inositol 5-phosphatase CIL-1 is required for normal PKD-2::GFP localization [4]. C. elegans NPHP-1 and NPHP-4 are required for cilia development and morphology in PKD-2 expressing neurons [5]. In C. elegans sensory neurons, MKS-3 localizes to the ciliary base. In an mks-3 mutant, PKD-2::GFP accumulates abnormally at the cilia base. These findings suggest that these ciliary base-localized proteins might play a general role in regulating ciliary protein localization. We are therefore examining interactions among these genes regarding to ciliary protein localization regulation. 1 Bae YK et al 2008 Dev Dyn 237:2021-9. 2 Bielas SL et al 2009 Nat Genet. 41:1032-6. 3 Jacoby M et al 2009 Nat Genet. 41:1027-31. 4 Bae YK et al 2009 The CIL-1 phosphoinositide 5-phosphatase regulates ciliary localization of the TRP polycystins and sperm function in C. elegans. Current Biology. In Press. 5 Jauregui AR et al 2008 J Cell Biol180: 973-88.

136/B83
New Roles for Ciliary Kinesins in Male-Specific CEM Sensory Neurons.
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The cilium is a specialized organelle used by a cell to detect and convert extracellular sensory stimuli into appropriate intracellular responses. Although all cilia are built by a conserved kinesin-2-driven process, termed intraflagellar transport (IFT), their structural and functional diversity is conferred by cell-specific modulation of IFT by additional motors and/or signaling proteins. Our objective is to explore the roles of multiple kinesins in specializing cilium length and shape in C.elegans nematode. We employed In Vivo fluorescent microscopy to examine CEM cilia morphology in wild-type males and mutants defective in the IFT kinesins KLP-11 and OSM-3 and male-enriched kinesin-3 KLP-6. Cilia axoneme reporter β-Tubulin-4::GFP shows that cilia length is similar in wild-type, osm-3(p802) and klp-6(my8) males (~3.4µm). Surprisingly, klp-11(tm324)
mutation resulted in changed shape and elongation of the CEM cilia to 5±9µm. Double mutant klp-11 osm-3 males had variable phenotype with missing, short or long CEM cilia. Additional removal of KLP-6 (in klp-11 osm-3; klp-6 triple mutants) suppressed the long cilia category and resulted in stunted or absent CEM cilia. These results provide important insight to mechanisms controlling ciliary length and indicate that a) OSM-3 is not required for CEM cilia formation and maintenance, b) KLP-11 and OSM-3 act partly redundantly in CEM cilia biogenesis, and c) KLP-6 acts with the IFT kinesins to regulate CEM ciliary length. We hypothesize that KLP-6 may regulate ciliary length via vesicular transport-mediated delivery of membrane to the ciliary base. Additionally, we propose that KLP-11 plays a CEM cilia-specific role in length restriction and shape definition. We are utilizing In Vivo time-lapse imaging to measure IFT particle velocity using cargo protein OSM-6::GFP to assess the effect of klp-11, osm-3 and klp-6 mutations on dynamics of intraflagellar transport in CEM cilia.

137/B84
Systems-Level Control of Longevity in Yeast: Aging Is a Developmental Program.
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The yeast Saccharomyces cerevisiae is a valuable model for unveiling the mechanisms of cellular aging in multicellular eukaryotes. Yeast aging can be slowed down by calorie restriction (CR), a low-calorie dietary regimen that extends life span in a wide spectrum of organisms and delays the onset of age-related disorders in rodents. To define a specific pattern of metabolism and organelle dynamics that is responsible for the anti-aging effect of CR and to establish the mechanisms underlying such effect, we assessed the effect of a CR diet and numerous mutations extending life span on the age-dependent dynamics of cellular and organelar proteomes and lipidomes, carbohydrate and lipid metabolism, interorganellar metabolic flow, concentration of reactive oxygen species, frequencies of nuclear and mitochondrial DNA mutations, mitochondrial morphology, stress response, and apoptosis. We show that yeast merge a number of cellular processes, which we call modules, into a metabolic longevity network. Our findings suggest a model for the spatiotemporal dynamics of this network. This model envisions that 1) yeast establish a diet- and genotype-specific configuration of the network by setting up the rates of the processes taking place within each of its modules; 2) the establishment of a network’s configuration occurs before yeast enter a non-proliferative state; and 3) different network’s configurations established prior to entry into a non-proliferative state define different rates of survival following such entry. Thus, by designing a specific configuration of the metabolic longevity network prior to reproductive maturation, yeast define their life span. We concluded that the chronological aging of yeast is a developmental program. Implementing our knowledge and using systems biological analysis, we identified five groups of novel anti-aging small molecules that extend yeast longevity by targeting 1) mitochondrial fusion and mitochondria-controlled, age-related apoptosis; 2) the protein kinase Rim15p, an essential component of the evolutionarily conserved nutrient-signaling network governed by protein kinase a and TOR complex; and 3) lipid metabolism in the endoplasmic reticulum, peroxisomes and lipid bodies.

138/B85
Adult-Onset Inhibition of SNF1A/dAMPKα Reduces Stress-Resistances and Shortens Lifespan in Drosophila Melanogaster.
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Energy homeostasis and stress resistance are closely linked to aging and longevity. AMPK (AMP-activated protein kinase), a sensor of cellular energy status, is activated by metabolic stresses that accelerates AMP/ATP ratio, regulating energy balance in whole organism. Recently AMPK is recognized as attractive therapeutic target for cancer, diabetic mellitus and obesity, providing a link to the metabolic syndrome. However little is known how functional deficiency of AMPK during adulthood affects aging and longevity In Vivo due to its redundancy and critical role.
in the development. Drosophila SNF1A/dAMPKα (CG3051) is a single orthologue for its mammalian counterparts. Using time- and tissue-specific RNAi system in Drosophila, we examined whether adult-onset dAMPKα RNAi affects lifespan and which tissue is responsible for its effect on longevity. We show that adult-onset inhibition of dAMPKα, especially in muscle, shortens lifespan. Further, inhibition of dAMPKα during adulthood enhances sensitivity to paraquat and starvation stress. Real-time PCR studies showed that adult-onset inhibition of SNF1A/dAMPKα decreased the expression levels of stress-resistant genes. Our results raise a possibility that dAMPKα could be one of master regulators integrating aging/longevity, metabolic hemostasis and stress resistance in whole organism.

139/B86

**Analysis of Yeast Lifespan Mediated by the Ca\(^{2+}\)-Signaling Pathways.**

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The \(zds1\Delta\) strain of the *Saccharomyces cerevisiae* shows the sensitivity to Ca\(^{2+}\) and the Ca\(^{2+}\)-induced, Swe1p- and Cln2p-mediated G2 cell cycle arrest, and polarized bud growth. To clarify a overview of the cell cycle and the growth control, we screened for suppressor mutants of the Ca\(^{2+}\)-sensitive phenotypes of \(zds1\Delta\) cells. Recessive mutants so far isolated have been classified into 14 genetic complementation groups (scz mutant). Of these mutants, we identified mutant allele of *SIR3* as *scz14*. In the recent research, it has been reported that Sir3 is involved in silencing of mating loci/telomeric, stress response, and aging. Therefore, we focused on the relationship aging with Ca\(^{2+}\)-signaling. Because other SCZ had been expected to be involved in aging, we examined the lifespan of *scz* mutants to understand the molecular mechanism by which Ca\(^{2+}\) involved in aging through the analysis of SCZ. The replicative lifespan in budding yeast is defined as the number mitotic cycles completed by a mother cell before senescence. We first examined the replicative lifespan of wild-type, \(zds1\Delta\), *swe1\Delta/scz1*, *mpk1\Delta/scz2*, *bck1\Delta/scz3*, *cnb1\Delta/scz4*, *ssn2\Delta/scz5*, *pkc/scz6*, *scz7*, *mck1\Delta/scz10*, *alg9\Delta/scz13*, and *sir3\Delta/scz14*. All *scz* strains had a shorter lifespan than wild-type, suggesting that SCZ is involved in the regulation of yeast lifespan. Next, we investigated the telomere length, known to be related to the lifespan, of these strains by Southern blot analysis. As a result, the telomere length of most strains were shorter than wild-type. These results suggested that SCZ genes are related to the aging and the telomere length. Thus, we proposed that Ca\(^{2+}\)-signaling is important for the regulation of aging and the maintenance of the telomere length.

140/B87

**Mechanical Characterization of Schlemm’s Canal Endothelial Cells In Vitro.**

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Background: The elevated intraocular pressure (IOP) associated with primary open angle glaucoma is caused by dysfunction of the tissues of the aqueous humor outflow pathway. However, the cellular and molecular basis of this dysfunction remains unknown. The bulk of aqueous humor outflow must traverse the endothelial lining of Schlemm’s canal (SC). We therefore hypothesize that the mechanical properties of the SC endothelium may contribute to the regulation of IOP. Objective: Systematic characterization of the mechanical properties of SC cells isolated from healthy donors. Methods: Using a micro rheological assay, optical magnetic twisting cytometry (OMTC), we focus on the baseline cell rheology, pharmacological responses to relaxing and contractile agonists, and how these measurements vary with In Vitro passage number. Results: Similar to other adherent animal cells in culture, the SC cells were found to exhibit weak power-law rheology with a median shear modulus of ~ 400 Pa (at 0.75 Hz), similar to that measured for other endothelial cell types. The baseline cell stiffness was nearly constant from passage 2 (p2) to p6; the contractile response to fetal bovine serum (1%) was constant from p2 to p4 but decreased appreciably in p5 and p6; the relaxing response to isoproterenol (100 uM)
were found the same between p2 and p3, but decreased in p4, p5 and p6. We also report the
dose response for contractile/relaxing agonists, including LPA, histamine, isoproterenol, and Y-
27632. Conclusion: Our measurements begin to define the mechanical phenotype of SC cells In
Vitro and could contribute to a better understanding of the cellular basis of glaucoma
pathogenesis.

141/B88
The Golgi Protein Gorab Links the Hereditary Disease of Gerodermia Osteodysplastica to
the Intracellular Trafficking Machinery.
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Wrinkling of the skin and reduction in bone density are natural consequences of aging. Since
osteoporosis is a severe health problem impeding the life quality of an ever increasing number
of elderly people it is of utter importance to understand the intracellular and physiological aspects of
bone formation and maintenance. Gerodermia osteodysplastica (GO) is an autosomal recessive
disorder characterized by wrinkly skin due to elastic fibre defects and osteoporosis with increased
fracture rate. It is caused by loss-of-function mutations in GORAB (golgin, RAB-6 interacting),
which is most prominently expressed in skin and osteoblasts (Hennies HC et al, Nature Genetics
40, 2008). The protein GORAB localizes to the Golgi apparatus and has been shown to interact
with the small GTPase RAB6 (Ras-related in brain) by yeast 2-hybrid and In Vitro pulldown
assays. This identifies GORAB as a golgin. In further assays we found evidence for an interaction
with the small GTPase ADP-ribosylation factor 5 (ARF5), which is also Golgi localized.
Knockdown of neither RAB6 nor ARF5 causes GORAB to dissociate from the Golgi apparatus.
The interaction of RAB6 maps to the central part of GORAB and the in silico identification of a
GRIP-like domain in the C-terminus, known to relate interaction with ARF- like 1 (ARL1) or ARF1
(Panic B et al, Mol Cell. 12, 2003; Gillingham AK et al., J Cell Biol. 167, 2004), hint at an
intriguing mechanism of cooperative binding of these two small GTPases. Electron and
epifluorescence microscopy showed that acute knockdown of GORAB in HeLa cells causes a
mild disruption in the Golgi structure. While Golgi morphology in non-confluent cultured skin
fibroblasts from GO patients was not altered, the Golgi structure became disturbed when the cells
reached confluency. In this state, cells secrete high amounts of extracellular matrix and the
intracellular trafficking machinery is highly taxed. These findings show that disturbance of Golgi
function and intracellular trafficking can cause diseases with aberrations of the extracellular
matrix in skin and bone.

142/B89
They Will Survive: Novel akt Mutants Have Extended Lifespan during Amino-Acid
Starvation.
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The protein kinase akt, a central component of the insulin signalling pathway, has been
implicated in the control of cell growth and cell survival through a variety of mechanisms,
including the negative control of apoptotic targets. Up-regulation of akt leads to an increase in
growth, while reducing akt activity decreases growth, and can be lethal. Several Drosophila akt
mutant lines were created through the mobilization of a P-element located within the upstream
control region of the gene. Characterization of the mutants showed variability in development and
overall growth. The time required for the mutants to reach eclosion is delayed when compared to
tROLS. Consistent with previously analyzed hypomorphic alleles, analysis of both mutant eyes
and somatic clones of the eye reveal that altered akt activity leads to a reduction in cell number
and size. Interestingly, these mutants have a moderate decrease in lifespan when aged upon
standard media, yet they show an increase in lifespan upon amino-acid deprived media when compared to the control. Replacement of akt activity in the mutants is sufficient to suppress this extension in lifespan, indicating it is the reduction in akt activity which produces this phenotype. These experiments can lead to a better understanding of the mechanisms that control survival during conditions of starvation, which can aid in understanding the progression of diseases which affect feeding behaviours, such as anorexia and other eating disorders. Support contributed by an Natural Sciences and Engineering Research Council of Canada (NSERC) PGS-D3 to J.D. Slade and an NSERC Discovery Grant to B.E. Staveley.

143/B90
Epigenetic Regulation of iPS Reprogramming in Premature Aging Mouse Model–Zmpste24 Deficient Mice.
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Zmpste24−/− mouse is a premature aging mouse model for human Hutchinson-Gilford progeria syndrome (HGPS). Zmpste24−/− MEFs and fibroblasts from HGPS patients are more sensitive to DNA-damaging agents and exhibit defective repair and delayed checkpoint response. HGPS cells and normal aged cells show decreased levels of H3K9 trimethylation and increased levels of H4K20 methylation. In addition, trimethylation of H3K27 is reduced in female HGPS cells. Our recent data indicates that acetylation of H4K16 is decreased in Zmpste24−/− MEFs. All these findings drove us to ask whether accelerated senescence could be a barrier to iPS reprogramming and whether the epigenetic changes observed in Zmpste24−/− MEFs could be reprogrammed. I utilized fibroblasts derived from Zmpste24−/− mice to carry out the iPS induction by introducing c-Myc, Klf4, Oct4 and Sox and found similar induction efficiency of reprogramming between Zmpste24−/− and wild-type MEFs. Furthermore, H4K16 acetylation in Zmpste24−/− MEFs derived IPS was found to be similar to wild-type MEFs derived iPS. These data provide an explanation for the similar efficiency between aging cells and wild-type cells and suggest that aging associated epigenetic changes can be reprogrammed during the iPS induction.

144/B91
Tenascin C Protects Aortic Tissue from Multiple Insults against the Development of Aortic Aneurysm.
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Aortic aneurysm (AA) is a common disease in the elderly people, caused by a segmental weakening of the aortic walls, which leads to the progressive aortic dilation under hemodynamic stress and eventually causes sudden death by the rupture of aorta. Recently, we reported that c-Jun N-terminal kinase (JNK) works as an integrator of multiple insults on aortic walls and regulates tissue response toward the destruction. Little is known, however, about the protection mechanism of aortic tissue against the development of AA. Tenascin-C (TN-C) is a member of large oligomeric extracellular glycoprotein family that regulates the cell migration, inflammation and fibrotic changes and healing of the tissue. In the current study, we tested the hypothesis that TN-C protects aortic tissue from the development of AA. We found that TN-C is expressed in aortic walls where the tissue destruction is most active both in human AA and in mouse model of AA. Systemic infusion of angiotensin II (AngII) and infrarenal periaortic application of CaCl2, representative insults to induce AA in mice, induced systemic and local expression of TN-C, respectively, in aorta. Simultaneous application of systemic AngII and infrarenal CaCl2 enhanced the suprarenal expression of TN-C in aorta, suggesting this segment of aorta experiences greater stress upon the combined insults than a single insult. Strikingly, combined insults with AngII and CaCl2 caused the development of large aneurysm in the suprareal aorta that resembles the
human disease only in TN-C knockout mice, while single treatment either with AngII or CaCl2 showed comparable effect on wild type and TN-C knockout mice. Transcriptome analysis showed that TN-C suppresses the inflammatory mediators and elastolytic enzymes in vascular smooth muscle cells. Therefore, TN-C is an integrator of multiple tissue insults that protects the aortic walls from the development of AA.

145/B92
Towards a Molecular Understanding of the Association of Desmin and Nebulin Filaments.
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Desminopathies constitute a newly identified myopathy manifesting a wide range of symptoms that are frequently present in cardiac sudden death. Over 45 mutations in desmin have been identified in humans as causative for desminopathy, characterized by dissolution of myofibrils, streaming Z-discs, and accumulation of protein aggregates. Desmin, the main intermediate filament (IF) protein in striated muscle, binds to nebulin establishing a direct link with the basic muscle contractile unit the sarcomere. Our studies aim to temporally elucidate the binding of desmin to nebulin during distinct stages of IF assembly. Our results show that multiple desmin domains bind to a high-affinity nebulin fragment encompassing modules 160-164 by blot overlay assays, but displayed no binding to keratins or lamins. Consistent with these findings, our in Vitro assembly and cosedimentation assays demonstrated that the nebulin fragment was pelleted with desmin headless and tailless truncation constructs but to a lesser extent than with rod domain, indicating that the rod domain is important for the interaction. Electron microscopy revealed that this nebulin fragment integrated into the mature desmin filaments. Analysis by cosedimentation assays of the binding of mutant desmin E245D to nebulin, a residue previously shown to interfere with the ability of nebulin to precisely regulate thin filament lengths, revealed a strong binding as compared to wild-type protein. Taken together, our data suggest that the binding site of nebulin for desmin is not a single, linear binding site but instead encompasses multiple residues, and that the E245D mutation appears to alter the in Vitro binding of nebulin for desmin filaments. We propose a working model to explain our data by which desmin wraps around the Z-disc and binds to nebulin with multiple residues, some of which are buried and exposed upon stretch during actinomyosin activation.

Host-Pathogen Interactions I (146 – 163)

146/B93
NS1 of H5N1 Interacts with SAP-97 in a PDZ-Dependent Manner to Disrupt Epithelial Barrier Integrity.
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The ability of influenza a virus to cause global pandemics has been a great concern throughout history. Since the beginning of the H5N1 outbreak in 1996, there have been 436 reported human cases resulting in 262 deaths. The pathology of H5N1 infection includes pulmonary edema and diarrhea. Large scale sequencing of avian influenza a viruses revealed that nonstructural protein 1 (NS1) contains a class I PDZ motif. The NS1 proteins of influenza viruses of avian origin contain the PDZ motif ESEV which has been found to bind several PDZ domain proteins, while NS1 of human influenza viruses contain the PDZ motif RSKV that does not bind PDZ proteins. The interaction of NS1 and host proteins via the PDZ motif, is a determinate in the virulence of influenza viruses of avian origin. It is our hypothesis that the NS1 protein of H5N1 increases
pathogenicity by interacting with host proteins through the PDZ motif. We are the first to show that the NS1 protein of A/chicken/Vietnam/C58/04 binds synapse associated protein-97 (SAP-97), an adherens junction protein, in a PDZ motif-dependent manner. In H5N1 infected tissues, the SAP-97 distribution is reorganized as evidenced by fluorescent immunostaining. Functionally, the interaction of NS1 and SAP-97 results in the loss of epithelial barrier function as measured by calcium switch and inulin flux assays. Disease pathology of the tissues of mice infected with H5N1 is more severe when infected with virus in which the NS1 protein contains the PDZ motif than those infected with virus which lacks the NS1 PDZ motif. The interaction of NS1 and SAP-97 helps explain the mechanism of why the two disease states, pulmonary edema and diarrhea, in which epithelial barrier is compromised, are both common in human H5N1 infection.

147/B94
Role of L-SIGN and DC-SIGN for Entry of Junin Arenavirus into Host Cells.
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Target cell tropism of enveloped viruses is regulated by interactions between viral and cellular factors during its replication cycle. Binding of viral envelope glycoproteins to specific cell-surface receptors determines susceptibility to viral entry. The entry and dissemination of viruses in several families can be mediated by C-type lectins such as hDC-SIGN and hL-SIGN. DC-SIGN is expressed in dendritic cells and has been identified as a receptor for different enveloped viruses. We used JUNV GPC pseudotyped particles or JUNV to study their ability to be internalized by hDC- or hL-SIGN. Results from transduction with JUNV pseudovirus show that infection of relatively non-permissive CHO cells was markedly enhanced when we over expressed hDC or hL-SIGN. Experiments using non-permissive mouse 3T3 cells showed that they become susceptible to JUNV pseudovirus transduction when they stable express DC-SIGN, or its homologue hL-SIGN, and that transduction of 3T3 stable expressing hDC-or hL-SIGN is blocked by anti-hDC/hL-SIGN antibodies and mannan. In addition, transduction of permissive CRFK was also enhanced when they were stable expressing DC-SIGN. Immunofluorescence assays with JUNV showed that infection of Vero cells was significantly enhanced by the over expression of hDC-SIGN or hL-sign, whereas infection of non-permissive murine cell line, 3T3, can be rescued by the expression of hDC-or hL-SIGN. Mannan treatment considerably reduced infection, in immunofluorescence and PFU assays, of lectin expressing cell lines, whereas anti-hDC/hL-SIGN antibodies showed reduction of infection as seen by PFU assay. Therefore DC- and L-SIGN can act as a novel attachment factors that mediate entry of JUNV. However, a number of cell-surface molecules bind viral envelope glycoproteins without mediating entry. Instead, they serve as capture receptors that disseminate viral particles to susceptible cells. We also demonstrate that the human C type lectins hL-SIGN and hDC-SIGN enhances transduction by JUNV pseudovirus particles in cells devoid of the JUNV receptor, hTfR1, indicating that C-type lectins can mediate virus entry in the absence of the virus cellular receptor.

148/B95
The HCV IRES Is a Potent Activator of PKR but Resistant to Eif2 Phosphorylation.
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Translation of hepatitis C virus (HCV) is initiated at an internal ribosome entry site (IRES) located at the 5’end of its RNA genome. The HCV IRES is highly structured and greater than 50% of its nucleotides form based-paired helices. We report here that the HCV IRES is an activator of PKR, an interferon-induced enzyme that participates in host cell defense against viral infection. Binding of HCV IRES RNA to PKR leads to a greatly increased (20-fold) rate and level (4.5-fold) of PKR autophosphorylation compared to previously studied dsRNA activators. We have mapped the domains in the IRES required for PKR activation to domains III-IV and demonstrate that the N-
terminal double-stranded RNA binding domains of PKR bind to the IRES in a similar manner to other RNA activators. Addition of HCV IRES RNA inhibits cap-dependent translation in HeLa S10 lysates via phosphorylation of PKR and elf2α. However, HCV IRES-mediated translation is not inhibited by the phosphorylation of PKR and elf2α. The results presented here suggest that hydrolysis of GTP by elf2 is not an essential step in IRES-mediated translation. Thus, HCV can use structured RNAs to its advantage in translation, while avoiding the deleterious effects of PKR activation.

**149/B96**

*Regulation of HIV Protein Levels by Two Cellular Pathways Provides New Insight into Control of HIV Replication.*

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Human immunodeficiency virus (HIV)-1 Rev is essential in the late phase of viral gene expression and replication. It regulates nuclear export, translation and encapsidation of Rev-response element (RRE) containing HIV mRNAs. In a proteomic study we have recently identified 95 human proteins that selectively interact with Rev and the RRE in vitro. Based on a probable role in RNA trafficking and other functions, we have selected a group of 17 proteins for further study in RNAi experiments. Silencing eight of these proteins caused specific loss of Rev functions in a Rev dependent Gag expression reporter assay. We have selected three of these: the E3 ligase Huwe1 and the RNA binding proteins ILF2 and ILF3. We have shown that these proteins are required not only for Rev functions, but also for viral replication, as silencing these proteins resulted in a 10 to 20 fold drop in p24 protein levels in HIV transfected cell supernatants. Surprisingly, the loss of Rev functions in siRNA knockdowns of Huwe1 and ILF2/3 is a result of the loss of the Rev protein itself. In each of these cases cytoplasmic Rev mRNA remains intact, however, Rev loss occurs through two distinct pathways. A Rev mutant where the lysine residues are replaced by arginine can rescue Rev dependent Gag expression in Huwe1 silenced cells, but not in ILF2/3 silenced cells. This suggests that Huwe1 regulates Rev levels via a ubiquitin related mechanism, whereas ILF2/3 regulation of Rev follows a different route. The description of the molecular mechanisms of Rev regulation via the Huwe1 and ILF2/3 proteins offers important insight into HIV replication and might eventually open up novel therapeutic avenues.

**150/B97**

*Streptococcal Collagen-Like Surface Protein 1 Promotes Adhesion to the Respiratory Epithelial Cell.*

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Collagen-like surface proteins Scl1 and Scl2 on Streptococcus pyogenes contain contiguous Gly-X-X triplet amino acid motifs, the characteristic of human collagen structure. The potential role of Scl1 in adhesion has been studied but yielded conflicting results. To explore the role of Scl1 in adherence to epithelial cells, we constructed a non-polar scl1 isogenic mutant from Scl2-defective S. pyogenes strain and a Scl1-expressed Escherichia coli. The loss of Scl1 resulted in a three-fold decrease in binding to human epithelial cells, HEP-2. Expression of Scl1 on the surface of a heterologous host E. coli significantly increased adhesion to HEP-2. Adhesion was abrogated when Scl1-expressed E. coli was pre-incubated with proteases or antibodies against recombinant Scl1 (rScl1) protein. Treatment of HEP-2 cells with rScl1 protein or pronase drastically reduced the binding capability of Scl1-expressed E. coli. These suggest that the adhesion is mediated through Scl1 on bacterial surface and protein receptor(s) on epithelial cells. However, blocking of potential integrins did not affect Scl1-mediated adhesion of bacteria to epithelial cells. Together, these
results underscore the importance of Scl1 in the virulence of S. pyogenes and imply that Scl1 may act as an adhesin during pathogenesis of streptococcal infection.

151/B98
Er Stress-Induced Conversion of Brucella-Replicative Organelles into Late Endosomal Vacuoles.

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Following uptake by phagocytes or entry into non-phagocytic cells, the intracellular pathogen Brucella abortus resides within a membrane-bound compartment, the Brucella-containing vacuole (BCV). The maturation of the BCV requires the Type IV secretion system VirB as a means of establishing a replicative niche in the endoplasmic reticulum (ER). During maturation the intermediate BCV traffic along the endocytic pathway and interacts with lysosomes, a process required to induce VirB functions and promote trafficking towards the ER. Although these stages of the intracellular cycle have been well characterized, little is known about post-replication events and how bacterial egress may occur. Here we show that at late times post-infection (2-3 days) in both primary macrophages and HeLa cells, Brucella infection induced ER stress via the Unfolded Protein Response (UPR). Concomitantly, BCVs re-acquired LAMP-1 positive endosomal membranes, suggesting a reassociation with the endocytic pathway, but did not display the autophagy marker MAP-LC3. Instead, this relocation was enhanced in either Atg5 -/- macrophages or HeLa cells treated with siRNA directed against both Atg5 and MAP-LC3, suggesting that Atg5 and LC3-dependent stages of autophagy interfere with the formation of these vacuoles. Characterization of the trafficking kinetics and identification of the host factors involved in Brucella survival late in infection will allow us to not only further characterize the pathway used by the bacteria to subvert normal host defense, but also understand the bacteria’s ability to egress, reinfect neighboring cells, and ultimately establish a chronic infection.

152/B99
FipA Is a Francisella tularensis Surface Protein Required for Intracellular Replication and Survival in Murine Macrophages.

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Francisella tularensis is a faculative intracellular pathogen that causes tularemia, a zoonotic disease with potentially lethal consequences in humans. The ability of F. tularensis to reside within host phagocytes is essential to the bacterium’s pathogenesis. Following phagocytosis, F. tularensis rapidly escapes its original phagosome into the host cytosol where it replicates extensively. Transcriptional profiling of the highly virulent F. tularensis subsp. tularensis strain Schu S4 at different stages of its intracellular cycle in murine bone-marrow derived macrophages (BMMs) revealed novel upregulated genetic determinants required for intracellular growth in macrophages and virulence in mice (Wehrly et al., 2009, Cell. Micro., 11(7):1128). One of these determinants, FipA (for Francisella lipoprotein A; FTT1676), encodes for a 330 amino acid with a putative lipoprotein signal sequence. Deletion of FipA in Schu S4 delayed phagosome disruption. ΔFipA mutant bacteria were replication deficient even after eventual escape into the host cytosol. Ultimately, a portion of the bacteria were found in membrane bound compartments labeled with the late endosomal marker LAMP1 and the autophagosomal membrane protein LC3, indicative of killing by an autophagy mediated mechanism. Immunofluorescence microscopy, surface protein biotinylation, and cell fractionation studies show that FipA localizes to the surface of Schu S4 during macrophage infection. Exposure on the bacterial surface situates FipA where it could potentially interact with the macrophage cytosolic compartment to impact the intracellular fate of F. tularensis.

153/B100
A Family of Salmonella Virulence Factors Functions as a Novel Class of Autoregulated E3 Ubiquitin Ligases.
Salmonella encodes at least two Type III Secretions systems, which function to deliver bacterial effector proteins into the cytosol of host cells. These effector molecules modulate the activities of several host signaling pathways leading to virulent infection. Here we show that the Salmonella effector protein SspH2 belongs to a growing class of bacterial effector proteins that harness and subvert the eukaryotic ubiquitination pathway. SspH2 possesses ubiquitination activity that is dependent on a conserved cysteine residue. A crystal structure of SspH2 reveals a canonical leucine rich repeat (LRR) domain that interacts with a unique E3 ligase (which we have termed NEL for Novel E3 Ligase) C-terminal fold distinct to that of previously observed HECT or RING-finger E3 ligases. Interestingly, the LRR domain partially occludes the catalytic cysteine residue contained in the NEL domain, and our data suggests that a substantial conformational change is required for optimal E3 ligase activity. In addition, we show that the N-terminal domain targets SspH2 to the plasma membrane of polarized epithelial cells, and propose a model whereby binding of the LRR to proteins at the target site releases the ligase domain for site-specific function.

154/B101 Subtilase Cytotoxin Cleaves Newly Synthesized Bip and Blocks Antibody Secretion in B Lymphocytes.
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Shiga-toxigenic Escherichia coli (STEC) uses subtilase cytotoxin (SubAB) to interfere with adaptive immunity. Its inhibition of immunoglobulin secretion is both rapid and profound. SubAB favors cleavage of the newly synthesized immunoglobulin heavy chain-binding protein (BiP) to yield a C-terminal fragment that contains BiP’s substrate binding domain. In the absence of its regulatory nucleotide binding domain, the SubAB-cleaved C-terminal BiP fragment remains tightly bound to newly synthesized immunoglobulin light chains, resulting in retention of light chains in the endoplasmic reticulum (ER). Immunoglobulins are thus detained in the ER, making impossible the secretion of antibodies by SubAB-treated B cells. The inhibitory effect of SubAB is highly specific for antibody secretion, because other secretory proteins such as IL-6 are released normally from SubAB-treated B cells. Although SubAB also causes BiP cleavage in HepG2 hepatoma cells, (glyco)protein secretion continues unabated in SubAB-exposed HepG2 cells. This specific block in antibody secretion is a novel means of immune evasion for STEC. The differential cleavage of newly synthesized versus ‘aged’ BiP by SubAB in the ER provides insight into the architecture of the ER compartments involved.

155/B102 Essential and Selective Interaction of the Chlamydial Inclusion with the Transferrin Recycling Pathway.
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Chlamydia is an obligate intracellular pathogen that resides within a parasite-defined vacuole termed an inclusion. The inclusion has selective interactions with host vesicular transport pathways, but the significance of these observations remains unclear. For example, a prominent feature of the chlamydial inclusion is its proximity to transferrin (Tf)-containing vesicles and the recycling endosomal markers Rab4 and Rab11, but there are no data demonstrating the essentiality of these interactions. We demonstrate that the Tf-containing vesicles surrounding the chlamydial inclusion are of the slow pathway of Tf recycling. Inhibitors known to arrest or delay this arm of Tf recycling were found to be potent inhibitors of chlamydial growth. In addition, quantitative analysis of Tf transport revealed that the inhibitors prevented the transport of Tf from
Rab11 vesicles to the Rab4/Rab11 intermediate compartment. This observation implied a requirement for the hybrid compartment in chlamydial growth. Indeed the apparent requirement for the Rab4/11 hybrid vesicle was confirmed by the observed partial growth inhibition of chlamydiae in cells co-expressing the dominant negative forms of Rab4 and Rab11. This report is the first demonstration of the biological relevance of and specific interactions with the TF recycling pathway by the inclusion. Findings provide insight into possible non-siderophore dependent mechanism of iron acquisition by this intracellular pathogen.

156/B103
A Novel Method to Track the Dynamic Interplay between Bacterial and Host Factors during Pathogen-Induced Vacuolar Rupture in Real Time by Fluorescence Microscopy.
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A common feature of the infection of eukaryotic cells by pathogens is their capacity to hijack host cellular processes. Gram-negative bacteria such as Shigella flexneri and Salmonella enterica are able to trigger their own entry via the needle-like type three secretion system. The formation of a bacteria containing endocytic vacuole within the cytosol of host cell is the subsequent and common step for both pathogens after internalization. Shigella escapes very quickly from this niche to reach the cytosol within minutes, however Salmonella remains multiple hours inside the vacuole forming filamentous structures called "sifs". In order to decipher molecular events involved in the rapid rupture of Shigella’s vacuole, we developed a novel approach based on fluorescence microscopy allowing us to follow the escape of Gram-negative pathogens from a vacuolar compartment into the cytosol with very accurate spatial and temporal resolution. Using small chemical inhibitors that interfere with host cellular processes, we show that the dynamics of vacuolar rupture by Shigella can be modified. This indicates the involvement of the host cell in this process. Concerning this, it is also of note that no bacterial factor has been identified to be directly involved in the rupture of the vacuole. Performing high-content/high-throughput approaches we are now delineating the precise molecular mechanisms exploited by Shigella to escape into the host cellular cytoplasm. Moreover, this will help finding new therapeutic target in order to develop drugs against pathogens infection.

157/B104
Limited Actin Proteolysis Mediates Invasion of Bacteria Serratia sp. in Eukaryotic Cells.
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Virulence of bacterial pathogens depends on their ability to attach and invade the host cell. Penetration of bacteria into eukaryotic cell is based on the interaction of bacterial factors with cytoskeleton and signaling system of the host. Earlier we have shown that synthesis of bacterial protease ECP32/grimelysin displaying specific activity towards actin correlates with the invasive activity of the host bacteria. Grimelysin from Serratia grimesii has been shown to be highly homologous to proteolysin from Serratia proteamaculans that differs from grimelysin by only 8 amino acid substitutions. In this work, we describe limited proteolysis of actin with proteolysin and invasive properties of the host bacteria S. grimesii DZMO 30063 and S. proteamaculans 94. Furthermore, taking into account that actin may be a natural substrate for these proteases, we address the question of a direct involvement of actin-specific proteases grimelysin and proteolysin in the process of bacterial invasion. To determine the role of grimelysin and proteolysin in capability of the bacteria to invade eukaryotic cells, human larynx carcinoma Hep-2 cells were incubated with recombinant bacteria E. coli K-12 and E. coli BL21DE3, expressing grimelysin and proteolysin genes, respectively. The cells were analyzed by confocal and electron microscopy. The noninvasive bacteria strains E. coli K-12 and E. coli BL21DE3 bearing plasmids without inserts of grimelysin or proteolysin gene were used as a control. Incubation of both
recombinant bacteria strains with Hep-2 cells affected confluence of the cells monolayer, the cells altered the shape, their membranes had numerous actin containing protrusions. The bacteria were found in cytoplasm of approximately 10% of cells. EM confirmed the presence of bacteria in cytoplasm, often located in vacuoles. In contrast, the control bacteria were not found in cytoplasm. The recombinant bacteria were further subjected to quantitative invasion analysis. In summary, expression of grimelesin or proteasins genes leads to appearance of invasive phenotype of normally noninvasive bacteria strains suggesting an active role of these proteases and limited actin proteolysis in the *Serratia* invasion.

158/B105
**Specific Innate Immunity: The Itam-Containing Ceacam3 Is a Molecular Mimic That Elicits Opsonin-Independent Activation of Neutrophil Bactericidal Responses against Neisseria gonorrhoeae.**

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Infection with *Neisseria gonorrhoeae* promotes a massive infiltration of polymorphonuclear neutrophils (PMNs) to the infected urogenital tract. The gonococci interact directly with these bactericidal leukocytes in an opsonin-independent manner, via interactions between bacterial Opa proteins and members of the human carcinoembryonic antigen related-cellular adhesion molecule (CEACAM) family. While PMNs from wild-type mice do not bind or engulf *N. gonorrhoeae*, PMNs from transgenic mice expressing human CEACAMs effectively bind, engulf, and mount a bactericidal oxidative burst and degranulation response. Using a transfection-based approach, we show that all Opa-binding CEACAMs of PMNs (CEACAM1, CEACAM3 and CEACAM6) allow PMN capture and engulfment of *N. gonorrhoeae*. However, only the immunoreceptor tyrosine-based activation motif (ITAM)-containing CEACAM3 elicits the neutrophil’s degranulation and oxidative burst responses. Bacterial binding to CEACAM3-triggers the phosphorylation of Syk tyrosine kinase and its downstream effector, Vav, which is a guanine exchange factor for Rho family GTPases. Since Vav has been implicated in a variety of neutrophil functions, we bred CEACAM transgenic mice with those lacking the Rac isoforms of neutrophils, Rac1 or Rac2, to discern the contribution of these proteins in the CEACAM3-dependent responses. Unexpectedly, we show that genetic ablation of either Rac1 or Rac2 in PMNs is not sufficient to abolish CEACAM3-dependent phagocytosis, oxidative burst or degranulation, suggesting either a redundancy in function or the involvement of another GTPase. We also show that CEABAC neutrophils, but not neutrophils from wild-type littermates, can produce the proinflammatory chemokines MIP-1 and MIP-2 in response to neisserial infection, providing an avenue for the rapidly progressing inflammation during gonorrhea. Consistent with this finding, a substantial defect in neutrophil influx is evident in CEACAM3-expressing mice exposed to *N. gonorrhoeae* in the absence of Rac. Together, these results define CEACAM3 as a molecular mimic of intercellular adhesion molecules commonly targeted by bacterial pathogens, allowing induction of a potent specific innate immune response by neutrophils.

159/B106
**The Rickettsia Outer Membrane Protein Sca2 Is an Actin Nucleator.**

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Many species of the bacterial genus *Rickettsia* utilize actin-based motility to move within and between infected host cells. *Rickettsia* actin tails contain long, unbranched actin filaments, in contrast to the short, branched networks generated by other intracellular pathogens. The current model of *Rickettsia* motility, in which the rickettsial protein RickA activates the host Arp2/3 complex, does not account for the unique ultrastructure of *Rickettsia* actin tails, the absence of RickA from the genome of the motile species *Rickettsia typhi*, or conflicting evidence regarding the localization of Arp3. These data suggest that additional motility factors remain to be defined. We searched *Rickettsia* genome databases for sequences with similarity to the well-characterized...
WH2 actin-binding motif and identified three such motifs in the outer-membrane protein Sca2 (surface cell antigen 2), which is present in all known motile *Rickettsia* species. Using In Vitro pyrene-actin polymerization assays, we show that the WH2-like part of Sca2 from *Rickettsia parkeri* binds actin monomers and inhibits actin filament assembly. Expressed separately, the N-terminal part of Sca2 promotes actin polymerization in vitro, binds filament barbed ends, and has distant sequence similarity to the formin family of actin nucleators. Expressed together, these two parts of Sca2 modulate actin filament dynamics, inhibiting assembly at lower Sca2 concentrations and promoting assembly at higher concentrations. Immunofluorescence microscopy reveals that Sca2, unlike RickA, is abundant on the surface of *R. parkeri* and is enriched at the actin-associated pole. These results suggest that multiple activities of Sca2 - monomer binding, filament nucleation, and protection of barbed ends - contribute to the formation of *Rickettsia* actin tails. We propose a new model in which Sca2, independently of RickA and the Arp2/3 complex, nucleates actin filaments and enables filament elongation during *Rickettsia* actin-based motility.

160/B107

*Alternaria alternata* Induces Ca$^{2+}$ Signaling and Cytokine and Growth Factor Release from Human Airway Epithelial Cells via the Protease Activated Receptor 2.

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From examination of the environmental exposures most closely tied to childhood asthma worldwide (housedust mite, fungi, cockroaches, and pet-related constituents), a concept is developing that these disparate entities with myriad different constituents do have a few commonalities, including the production of proteases with trypsin-like function. In this report we examine *A. alternata* filtrates and their ability to activate human bronchial airway epithelial cells (16HBE14o-) as measured by Ca$^{2+}$ signaling and proinflammatory cytokine and growth factor release. We show that *A.alternata* filtrates induce transient Ca$^{2+}$ changes consistent with activation of Ca$^{2+}$ release from intracellular stores. These Ca$^{2+}$ transients are blocked by heat inactivation, protease inhibitor cocktails and a serine-specific protease inhibitor (AEBSF) pretreatment of *A. alternata*. To more closely evaluate receptors involved in *A.alternata*-driven activation, we have developed a HeLa cell line that stably expresses the protease activated receptor 2 (PAR-2) protein. *A. alternata* filtrates produce Ca$^{2+}$ transients in PAR-2 transfected cells, whereas they are ineffective on untransfected HeLa cells. To better evaluate physiological responses to *A. alternata*, we repeated stimulation of 16HBE14o- and monitored cytokine and growth factor secretion. *A. alternata* filtrate stimulation of 16HBE14o- cells induced increased proinflammatory cytokine release (IL1$\alpha$, IL6, IL8, and TNFa) and growth factor release (GMCSF, GCSF, VEGF and FGF) within 3 hours. To better examine the role of PAR-2 in these responses, we used the PAR-2 peptide agonist, SLIGRL to activate the 16HBE14o- cells and noted a similar proinflammatory cytokine release pattern by 3 hours of incubation, however, only GMCSF and GCSF displayed significant increases among the growth factors. These data support the hypothesis that *A. alternata* proteases induce rapid increases in human airway epithelial intracellular Ca$^{2+}$ concentration and proinflammatory cytokine release that occurs in part via PAR-2 and that these responses may be critical early steps in the development of asthma.

161/B108

A Novel Mechanism for Generation of Antibodies against a Xeno-Auto-Antigen Involving Bacterial Scavenging and Expression of a Dietary Glycan.

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All humans have circulating antibodies against N-glycoly neuraminic acid (Neu5Gc), a sialic acid that cannot be synthesized by humans. Red meat and milk contain large amounts of Neu5Gc that, when ingested, can be metabolically incorporated into human tissues, especially carcinomas. Thus, the potential exists for circulating anti-Neu5Gc antibodies to react against endogenous Neu5Gc, which we call a "xeno-auto-antigen", as it is a foreign antigen that can become immunoreactive self. Indeed, we have recently demonstrated that anti-Neu5Gc antibodies contribute to chronic inflammation in the promotion of tumor progression. Here we show that anti-Neu5Gc antibodies appear in infants at 6-12 months of age and that their timing of appearance correlates with the introduction of Neu5Gc in the diet. The timing of the appearance of anti-Neu5Gc antibodies also appears coincident with antibodies against Nontypeable Haemophilus influenzae (NTHi), an obligate human commensal and pathogen that is a major cause of otitis media in infants and children. NTHi has been shown by others to scavenge N-acetylneuraminic acid (Neu5Ac), a sialic acid synthesized by humans, in order to mask it’s lipooligosaccharide (LOS) glycans and evade host immune attack. We show that, in addition to Neu5Ac, NTHi can also scavenge exogenously administered free Neu5Gc in vitro and express it on it’s LOS. Furthermore, we demonstrate that Neu5Gc-deficient mice infected with NTHi expressing Neu5Gc generate anti-Neu5Gc antibodies. Finally, purified human anti-Neu5Gc antibodies specifically recognize Neu5Gc expressing NTHi but not Neu5Ac-expressing or nonsialylated NTHi. Based on our findings we propose a novel model for the generation of human anti-Neu5Gc antibodies in which dietary Neu5Gc is taken up and expressed by colonizing bacteria, which provide the immunogenic stimulus required to produce antibodies against Neu5Gc.

162/B109

Antibody-Mediated Immune Responses against Crude Schizont Extract Are Comparable to Responses of AMA-1, GLURP, MSP1-19, and MSP3.

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Many blood-stage malaria vaccine candidate antigens have been identified and it is likely that a malaria vaccine candidate will consist of more than one candidate antigen. The objective of the study was to assess the immune responses to the crude schizont extract which contains many antigens, in relation to some blood-stage vaccine candidate antigens which are AMA-1, GLURP, MSP1-19 and MSP3. Plasma samples were obtained from a cohort of 352 children, aged, 3 to 10 years, in Ghana at the beginning of a nine-month morbidity surveillance which coincided with the beginning of the malaria transmission season. Crude schizont extract was prepared by continuously culturing the malaria parasites and harvesting the schizont-rich RBCs using the magnetic-activated cell sorting system and lysing the schizont-rich RBCs to release the parasite antigens into solution. The supernatant of the suspension contained the crude schizont extract. IgG and IgM responses against the crude schizont extract and the recombinant antigens were measured by indirect ELISA. The proportion of the participants that had asymptomatic parasitemia was fairly constant throughout the surveillance (50-65%), while the incidence of malaria rose from March, peaking in July and then with a general decline through November. The proportion of IgM and IgG responders to the four recombinant antigens and the crude schizont extract ranged from 30%-95% with that of schizont extract being 83% and 87% respectively. Antibody levels increased with age and protection with the median age for susceptible children (5 years) being significantly lower than that for protected children (8 years) (p=0.001). After adjusting for the confounding effect of age, the data showed a significant association between age and IgM and IgG responses to all the antigens tested with p-values between 0.002 and 0.03.
In conclusion, immune responses against the crude schizont extract which contains numerous antigens presented by the schizont at the blood-stage was comparable to AMA-1, GLURP, MSP1-19 and MSP3, and further studies must be done to confirm its correlation with protection.

163/B110
A Novel Erythrocyte Targeted Parasite Protein Is Important for the Survival of Plasmodium Falciparum.
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Plasmodium falciparum cause the most severe malaria called cerebral malaria, which accounts for more than 1.5 million deaths annually. In humans the malaria related clinical symptoms arises due to the erythrocytic cycle where the parasite resides in the parasitophorous vacuole inside the red blood cells. During the progression of the erythrocytic cycle the parasite extensively modifies the infected RBC (iRBC) by recruiting ~ 400 proteins in the iRBC cytoplasm and surface. Apart from few characterized exported proteins, majority of them are of unknown functions, and lack homology with any known proteins. Mature RBC completely lacks the transport pathway so to facilitate export of the proteins and nutrients the parasite develops extensive membranous structures in the iRBC cytoplasm spanning form the parasite confines to the RBC membrane called as Maurer’s clefts and Tubo-vesicular network (TVN). So far, no consensus motif has been found in the Plasmodium secretome and the mechanism of transport is very poorly known. In the present work we have identified a novel conserved membrane protein, PFI0160w previously known as hypothetical protein, which is expressed at early trophozoite stage and reaches its maxima in the schizont stage. By indirect immunofluorescence microscopy, electron microscopy and biochemical assays we have shown that this protein is exported from the parasite and localizes in the Maurer’s clefts before reaching to the iRBC surface. We have named it as “Parasite exported conserved membrane protein” (PEMP). The antibody against PEMP strongly inhibits the parasite growth with IC50 value of 40 μg/ml. The export of the protein is sensitive to brefeldin a (BFA) suggesting that it is exported following the classical secretory pathway. Our data projects PEMP as a potential drug as well as vaccine candidate against the deadly malaria pathogen.

Cell Biology of the Immune System I (164 – 174)

164/B111
FRET Reporters for Imaging MMP12 Activity as a Marker for Lung Inflammation.
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Chronic obstructive pulmonary disease (COPD) is accompanied by destruction of the extracellular matrix of alveolar airspaces of the lung and emphysema. It is believed that the destructive cause is an imbalance between proteases and antiproteases induced by an up-regulation of enzymes such as elastases and collagenases. A prime candidate is macrophage elastase also called matrix metalloprotease 12. Here we introduce a series of FRET-based peptide reporters for monitoring macrophage secreted MMP12 activity in lavage fluids from mice as well as on the surface of cells isolated form these lavages. To address the localization of the active enzyme on the cell surface, we prepared lipidated FRET-based reporters that reside on the plasma membrane. The synthesis of the peptidic backbone, the attachment of the fluorophores and the lipidation was achieved on a solid phase support. The lavage reporter LaRee1 inserted spontaneously into the plasma membrane of RAW macrophages and cells from lung lavages. Upon enzymatic cleavage, the acceptor was released but the lipidated donor fragment was sufficiently lipophilic to be internalized by endocytosis. The latter produced a memory signal that permitted us to identify activated macrophages in lung lavages of mice instilled with particulate matter, i.e. A model of acute pulmonary inflammation. The reporters are therefore useful to detect
MMP12 activity in pulmonary inflammation and may thus be useful to evaluate for emphysema formation at an early stage, thus providing important information for the diagnosis of lung diseases. In addition, we could demonstrate that secreted MMP12 activity resides exclusively on the surface of macrophages while MMP12 released into the supernatant or the lung lumen remained in the inactive pro-form. This implies that MMP12-induced damage to the extracellular matrix requires direct contact of macrophages which makes the mobility of these cells a prime target for a potential treatment for COPD.

165/B112
Overexpression of HO-1 Protects against TNF-α-Mediated Airway Inflammation by Down-Regulation of TNFR1-Dependent Oxidative Stress.
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Oxidative stresses are considered to play an important role in the induction of cell adhesion molecules and proinflammatory cytokines, a key event in inflammatory processes. Heme oxygenase-1 (HO-1) exerts as an antioxidant which protects against tissue injury. Here, we report that HO-1 was induced in cultured human tracheal smooth muscle cells (HTSMCs) by treatment with a potent inducer of HO-1 activity, cobalt protoporphyrin IX (CoPP IX), or infection with a recombinant adenovirus carrying the human HO-1 gene. Overexpression of HO-1 protected against TNF-α-mediated airway inflammation by down-regulation of oxidative stress, adhesion molecules, and IL-6 in HTSMCs and the airways of mice. HO-1 overexpression inhibited TNF-α-induced ICAM-1 and VCAM-1 expression, THP-1 cells adherence, IL-6 generation, p47phox translocation, and NF-κB activation. HO-1 overexpression also attenuated TNF-α-induced oxidative stress, which was abrogated in the presence of the HO-1 inhibitor, zinc protoporphyrin IX (ZnPP IX) and CO scavenger. In addition, HO-1 overexpression reduced the formation of a TNFRI/c-Src/p47phox complex. These results suggested that HO-1 functions as a suppressor of TNF-α signaling, not only by inhibiting adhesion molecules expression and IL-6 generation but also by diminishing intracellular ROS production and NF-κB activation in HTSMCs and the airways of mice.

166/B113
Amelioration of Asthmatic Inflammation by an Aqueous Extract of Spinacia Oleracea Linn.
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Inflammation of the respiratory tract is a crucial process in immune diseases, including asthma, and atopic rhinitis. To establish whether an aqueous extract of Spinacia oleracea Linn (SoL) has a beneficial influence in terms of anti-asthmatic activity, we examined its effects on an ovalbumin-induced asthmatic model. Mice sensitized to ovalbumin were orally administered the SoL extract, and their lungs examined by Haematoxylin-Eosin staining to determine IL-4/13 cytokine expression. The SoL extract exerted strong anti-asthmatic effects by inducing a decrease in the CD4+ cell number, IL-4/13, and other molecular markers in the lung. Our results collectively indicate that the aqueous SoL extract ameliorates asthmatic symptoms effectively in a mouse ovalbumin-challenge model.

167/B114
Caveolin-1 Regulates Monocytes Recruitment in Fibrotic Lung Disease.
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The reduced caveolin-1 level in scleroderma lung fibroblasts and the lungs of bleomycin-treated mice promotes collagen overexpression and lung fibrosis. We now evaluate whether caveolin-1 is also deficient in monocytes from bleomycin-treated mice and scleroderma patients and examine the consequences of this deficiency and its reversal. Mice or cells received the CSD (caveolin-
1scaffolding domain) peptide to reverse the pathological effects of reduced caveolin-1 expression. In bleomycin-treated mice, caveolin-1 expression was reduced and CXCR4 expression was increased in circulating monocytes. Systemic treatment with the CSD peptide reversed these effects as well as the accumulation of monocytes in lung tissue. To validate our results in human disease, we examined caveolin-1 and CXCR4 expression in circulating monocytes and lung tissue from scleroderma patients. We found that scleroderma monocytes contain less caveolin-1 and significantly more CXCR4 than their normal counterparts and that there is a massive increase in CXCR4 in the tissue of scleroderma patients with lung involvement. We also determined the molecular mechanisms regulating CXCR4 and caveolin-1 expression in scleroderma monocytes. The overexpression of CXCR4 in scleroderma monocytes is due to the low level of caveolin-1 in these cells as evidenced by the fact that CSD peptide treatment of scleroderma monocytes inhibits CXCR4 expression. The pro-inflammatory cytokine TNFα and the pro-fibrotic cytokine TGF-β cause normal monocytes to adopt the scleroderma phenotype: low caveolin-1 and high CXCR4. In summary, exposure of monocytes to TNFα and TGFβ reduces their caveolin-1 expression; in turn, reduced caveolin-1 expression upregulates CXCR4 expression. These results support the idea that using the CSD peptide to compensate for low caveolin-1 levels in scleroderma monocytes may be a useful treatment for scleroderma because this treatment will inhibit CXCR4 expression and thereby inhibit the accumulation of monocytes and monocyte-derived cells in injured lung tissue.

168/B115
Amelioration of Airway Inflammation via the Blockade of Lymphotoxin Signaling.
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Lymphotoxin (LT) has been known to be essential for IgE production, and a physiological role of IgE in the airway may consist of maintaining the balance of Th1 and Th2 responses to prevent aberrant inflammation. To investigate whether LT signaling involved in airway inflammation, anti-inflammatory reactions were examined by administration of LTbR-Ig from OVA (ovalbumin)/Alum (aluminium hydroxide) and HDM (house dust mite)-treated mice. The blockade of LT signaling by administration of LTb receptor (R)-Ig induced the reduction of the infiltration of inflammatory cells to airway. IgE levels in serum and bronchoalveolar lavage fluid (BALF) of those mice were markedly decreased by administration of LTbR-Ig compared with mice treated with human Ig. In addition, LTbR-Ig suppressed the expression of proinflammatory cytokines in the lung of OVA/Alum and HDM-treated mice. Furthermore, the LTbR-Ig-treated mice showed the ameliorated allergic symptoms in morphology. These results suggested that blockade of LTb receptor signals might be helpful to reduce the more increased airway inflammatory responses.

169/B116
TNF-α Decreases Aquaporin-3 Expression in Keratinocytes.
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[Objective] Aquaporin-3 (AQP3) is selectively expressed in the basal layer of keratinocytes in skin. Although AQP3 is believed to be important to maintain physiological hydration and wound repair in skin, expression or function of AQP3 in inflammatory conditions has not been understood. In the present study, we examined the effect of TNF-α, an inflammatory cytokine, on AQP3 expression in keratinocytes. [Methods] DJM-1 human keratinocyte cell line was cultured in MEM containing 10% FBS and antibiotics. The expression of AQP3 protein and mRNA was assessed with western blotting and real-time quantitative RT-PCR, respectively. AQP3 promoter (-990/+88) activity was measured by luciferase assay. [Results] TNF-α decreased the level of AQP3 protein in DJM-1 cells, in time- (0-24 h) and concentration- (0.02-20 ng/ml) dependent manners. The level of AQP3 protein in TNF-α treated (20 ng/ml, 24 h) cells was decreased to 50% of that in control cells. This decrease in AQP3 protein was correlated to the decrease in plasma membrane water permeability, assessed by stopped-flow method. TNF-α also decreased AQP3 mRNA and AQP3 promoter activity, without changing the stability of AQP3 mRNA,
suggesting that TNF-α decreased AQP3 transcription. The decrease in AQP3 by TNF-α was abolished SB203580 and PD98059, p38 and ERK inhibitors respectively, but not by CAPE, an NF-κB inhibitor. It is, therefore, suggested that TNF-α decreased AQP3 expression via MAPK-dependent pathways. [Conclusion] We demonstrated that TNF-α decreased AQP3 expression via ERK- and p38-dependent pathways. These data strongly suggests that AQP3 expression in keratinocytes may be decreased in inflammatory conditions. Considering the function of AQP3 in skin, TNF-α-induced decrease of AQP3 expression may be related to impaired skin hydration and wound repair in inflammatory conditions.

170/B117
Smad7 and Smad6 Use Distinct MH2 Subdomains to Bind to Discrete Regions of Pellino-1, Mediating TGF-Beta1-Induced Negative Regulation of IL-1R/TLR Signaling.
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The molecular mechanism by which transforming growth factor-beta (TGF-beta) suppresses inflammation is not well characterized. Here, we demonstrate that complete suppression of Interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) signaling requires the concerted efforts of TGF-beta-induced Smad6 and Smad7. Upon treatment of TGF-beta 1, Smad6 and Smad7 simultaneously bind to distinct regions of Pellino-1, an adaptor protein of interleukin-1 receptor associate kinase (IRAK1), via two different secondary structural elements in their MH2 domains. These interactions abrogate NF-kappaB activity by blocking formation of the IRAK1-mediated signaling complex downstream of IL-1R/TLR signaling. Double knock-down of endogenous Smad6 and Smad7 genes by RNA interference further reduced the anti-inflammatory activity of TGF-beta1 than when compared with single knock-down of either Smad6 or Smad7, suggesting that both Smad6 and Smad7 are required for maximal suppression of IL-1R/TLR signaling. Thus, we show that both Smad6(beta-sheet)and Smad7(alpha-helix) simultaneously bind to distinct regions of Pellino-1 via non-overlapping regions in their MH2 domains, and act as critical mediators for effective TGF-beta 1-mediated suppression of IL-1R/TLR signaling.

171/B118
Roles for the Protein Agrin in Regulating Inflammation In Vitro.
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Agrin is a heparan sulfate proteoglycan best known for its role in the organization and stabilization of numerous molecules, including acetylcholine receptors (AChR), during the formation of the neuromuscular junction. Interestingly, both agrin and AChRs are broadly expressed molecules, present in a variety of cells, including immune cells. Macrophages, phagocytes of the immune system that are primarily responsible for the eradication of infection, have been known to express AChRs and be involved in regulating inflammation. The cholinergic anti-inflammatory pathway is widely accepted as one mechanism of regulating inflammatory response. This pathway, termed the neuro-immune axis, is dependent on the acetylcholine (ACh) secreted by the vagus nerve. ACh inturn activates the α7 nicotinic acetylcholine receptors (α7-nAChRs) present on macrophages regulating the further production and synthesis of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) by inhibiting the nuclear factor kappa B (NF-xB) transcription factor. Using SDS-PAGE, immunoblotting, immunocytochemistry and ELISA techniques we find here that agrin increases α7-nAChRs expression in the macrophage cell line RAW264.7 and appears to aggregate surface α7-nAChRs in this cell line. Importantly, agrin alone does not appear to affect pro-inflammatory cytokine synthesis. The addition of ACh appears to diminish the effects of agrin, suggesting that agrin may play a role upstream of ACh signaling through α7-nAChRs. Together, these findings suggest a role for agrin in modifying an inflammatory response in the innate immune system by regulating the efficacy of vagus derived ACh.

172/B119
Inhibition of Cellular Response to Sphingosine-1-Phosphate by Human Plasma Gelsolin.
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Sphingosine-1-phosphate (S1P) is a potent and pleiotropic bioactive lipid mostly released by haematopoietic cells, that acts as an extracellular agonist and an intracellular second messenger. Cell activation by S1P requires precise control, as an acute increase in its plasma concentration is sufficient to induce sudden cardiac death through an S1P receptor-mediated mechanism. Here we report that human plasma gelsolin (hpGSN) acts as an S1P buffer, as it binds S1P and its synthetic structural analog (S)-FTY720-phosphate. The optical density at 280 nm of hpGSN decreased following the addition of S1P or (S)-FTY720-phosphate and hpGSN’s ability to depolymerize F-actin decreases progressively with increasing addition of phosphatidylinositol 4,5-bisphosphate (PIP2) and S1P at similar molecular ratios. Both the transient increase in phosphorylation of extracellular signal regulated kinase (ERK) and nuclear factor kB (NF-kB) cytosol-nucleoplasm translocation in bovine aortic endothelial cells (BAEC) after S1P treatment were inhibited by hpGSN. Evaluation of gelsolin and S1P levels in cerebrospinal fluid reveals a low concentration of gelsolin and high concentration of S1P in samples obtained from patients suffering from lymphatic meningitis and multiple sclerosis. These findings indicate that gelsolin-mediated buffering of S1P activity may be important to keep immunomodulatory balance at inflammatory sites.

173/B120
IL-1β-Activated Macrophages Stimulate Differentiation of Mesenchymal Stem Cells to Smooth Muscle Cells via Paracrine Mechanism.
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Mesenchymal stem cells (MSCs) have been known to be useful for regeneration of inflamed or injured tissues. Macrophages are involved in acute or chronic inflammatory diseases and IL-1β, a pro-inflammatory cytokine, plays a key role in the activation of macrophages. In order to explore the crosstalk between MSCs and macrophages within inflammatory environment, we examined the effects of conditioned media from macrophages in the differentiation potential of MSCs to vascular smooth muscle cells. We demonstrate here that IL-1β-conditioned media from macrophages (IL-1β CM) stimulate differentiation of MSCs to vascular smooth muscle cells, as evidenced by increased expression of smooth muscle-specific markers, including alpha-smooth muscle actin (α-SMA), smooth muscle-myosin heavy chain, and calponin. The IL-1β CM-induced expression of α-SMA was abrogated by pretreatment of MSCs with the MEK/ERK-specific inhibitor U0126. Moreover, pretreatment of MSCs with the Rho kinase-specific inhibitor Y27632 or over-expression of the dominant negative mutants of RhoA or Rho kinase blocked the IL-1β CM-stimulated α-SMA expression, indicating a pivotal role of ERK and Rho kinase in the IL-1β CM-induced differentiation of MSCs to SMCs. Taken together, these results suggest that IL-1β-activated macrophages promote differentiation of MSCs to vascular SMCs, which are crucial for vascular regeneration of injured tissues, through paracrine mechanism.

174/B121
Interleukin-1β Modulation of Intestinal Epithelial Tight Junction Barrier Requires Activation of p38 Kinase.
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Interleukin-1β (IL-1β) has been shown to play an essential role in mediating intestinal inflammation of Crohn’s disease (CD) and other inflammatory conditions of the gut. The IL-1β-induced increase in intestinal epithelial tight junction (TJ) permeability has been postulated to be an important pathogenic mechanism contributing to intestinal inflammation. However, the intracellular pathways that mediate the IL-1β modulation of intestinal TJ barrier function remain...
unclear. In these studies, we show that activation of p38 MAP kinase signaling cascade is required for the IL-1β induced increase in Caco-2 intestinal epithelial TJ permeability. The IL-1β caused a time-dependent increase in p38 activation as measured by phospho-p38 immunoblotting. Inhibition of p38 by specific inhibitor SB-203580 (25 μM) prevented the IL-1β induced drop in Caco-2 transepithelial resistance (TER) and also abolished the IL-1β induced increase in permeability to paracellular marker inulin. To further validate the role of p38 signaling pathway in mediating the IL-1β effect on Caco-2 TJ permeability, p38 expression was selectively knocked-down by transfection of p38 siRNA. The transfection of p38 siRNA resulted in a near-complete knock-down of p38 in filter-grown Caco-2 monolayers. The siRNA-induced knock-down of p38 prevented the IL-1β induced drop in Caco-2 TER and increase in inulin flux. IL-1β also caused a time-dependent increase in myosin light chain kinase (MLCK) activity and expression. Inhibition of MLCK by ML-7 (10 μM) prevented the IL-1β induced increase in Caco-2 TJ permeability. Moreover, inhibition of p38 by SB203580 prevented the IL-1β induced up-regulation of MLCK. p38 silencing by siRNA transfection also abolished the IL-1β induced increase in MLCK expression and activity. In conclusion, our data data suggest that the IL-1β modulation of intestinal epithelial TJ barrier is regulated by p38 signaling cascade induced up-regulation of MLCK activity and protein synthesis.

**Endothelial Cells (175 – 190)**

175/B122

**Developing New Angiogenic Models Based on a Novel, Stable, Fluorescently Labeled Endothelial Cell Line.**

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Formation of new blood vessels, or angiogenesis, has been implicated in many physiological processes and diseases and is, therefore, an important target for drug development. Recapitulation of vessel formation In Vitro by human cells not only allows investigating cell-cell and cell-matrix interactions in a controlled environment, but is also a crucial step in developing High Content Screening (HCS) assays that search for novel stimulators or inhibitors of angiogenesis. The typical cell type used in most angiogenic assays today is Human Umbilical Vein Endothelial Cells (HUVECs). However, because HUVECs are primary cells, their use is limited to 6-8 passages. To overcome this limitation, we have developed a 96-well HCS assay using HMEC-1, immortalized human microvascular endothelial cells (Ades et al., 1992). We showed that HMEC-1 cells, by themselves and in co-culture with Epicardial Mesenchimal Cells (EMC), are able to form branching networks on Matrigel. Moreover, we created HMEC-1 and EMC cell lines stably expressing nuclear-localized Histone-2B (H2B) fused with eGFP or mCherry fluorescent proteins, thus allowing easy cell tracking and screening by automated algorithms. We characterized the networks formed by these cells and showed them to be comparable to those formed by HUVECs in timing of vessel formation, number of branching nodes, branch length, and other angiogenic features. We propose to use these fluorescent HMEC-1 and EMC lines as a viable cell choice for future angiogenic assays.

176/B123

**Regulation of Endothelial Senescence by the Homeodomain Transcription Factors MEOX1 and MEOX2.**

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The mesenchyme homeobox genes, MEOX1 and MEOX2, encode homeodomain transcription factor proteins. In the adult, MEOX2 expression is maintained in the vasculature. MEOX2 is expressed in both vascular smooth muscle and endothelial cells where it blunts proliferation by inhibiting cell cycle progression. To date there are only two confirmed target genes of MEOX2;
the cyclin-dependent kinase inhibitors p21 CIP1/WAF1 (CDKN1A) and p16 INK4a (CDKN2A). The Meox1/Meox2 knockout mice have shown that the Meox proteins can partially compensate for one another during development; however the role of MEOX1 has yet to be studied in the vasculature. The objectives of our study were to: 1) Determine whether MEOX1 and MEOX2 play a role in endothelial cell senescence and 2) Identify novel target genes of MEOX1 and MEOX2 in endothelial cells. To fulfill these aims, the MEOX proteins were expressed in primary human umbilical vein endothelial cells (HUVEC) via adenoviral transduction. Expression of the p21 and p16 genes was measured by western blot and quantitative real-time PCR analysis. Cellular proliferation and senescence were assessed by analyzing bromodeoxyuridine (BrdU) incorporation and senescence associated beta-galactosidase (SA-β-gal) staining, respectively. Novel target genes were identified using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) expression microarray and verified via qRT-PCR. Both MEOX1 and MEOX2 activated p21 gene transcription in HUVECs. However, only MEOX2 induced a significant increase in p21 at the protein level. Surprisingly, the increased p21 expression induced by MEOX2 did not correlate with decreased BrdU labeling. MEOX1 and MEOX2 both activated p16 gene transcription in HUVECs which corresponded with increased SA-β-gal staining. Taken together, we conclude that both MEOX1 and MEOX2 are key regulators of cellular senescence in vascular endothelial cells.

Involvement of Rho-Kinase in LPA-Mediated Inflammatory Response through Activation of NF-κb and AP-1 in Endothelial Cells.

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Endothelial cells play an important role in the recruitment of immune cells through the induced expression of chemokines and cell adhesion molecules. The proinflammatory lipid mediator, LPA (lysophosphatidic acid) has been shown to induce IL-8, MCP-1, ICAM-1 and VCAM-1 expression in endothelial cells. However, the pathways involved in LPA-mediated inflammatory signaling in endothelial cells are not fully understood. The present study was undertaken to investigate the LPA-mediated inflammatory signaling in endothelial cells. LPA induced expression of IL-8, MCP-1, ICAM-1 and phosphorylated NF-κb p65 (Ser536), JNK/SAPK (Thr183/Tyr185), and c-Jun (Ser63) in HUVEC. LPA also increased c-Jun expression. Based on literature, linking LPA to ROCK activation, we examined the phosphorylation status of downstream substrates of Rho-kinase (ROCK), MLC (Thr19/Ser18) and MYPT1 (Thr850). LPA-mediated phosphorylation of MLC (Thr19/Ser18) and MYPT1 (Thr850) was blocked by a ROCK inhibitor (H-1152P) but not by a NF-κB inhibitor (Bay11-7082) or JNK inhibitor (SP600125). LPA-mediated phosphorylation of NF-κB p65 (Ser536) and translocation of NF-κB into nucleus were inhibited by H-1152P and Bay 11-7082, but not by SP600125. H-1152P and SP600125 inhibited LPA-mediated phosphorylation of JNK/SAPK (Thr183/Tyr185) and c-Jun (Ser63). LPA-induced c-Jun expression was inhibited by H-1152P but not by SP600125 and Bay 11-7082. In addition, H-1152P suppressed LPA-induced IL-8, MCP-1, ICAM-1, and VCAM-1 expression in HUVEC. These results indicate that ROCK plays a critical role in LPA-mediated inflammatory response through activation of NF-κB and AP-1 in endothelial cells.

Interleukine-23 Activates Endothelial Cells Causing Intracellular Signalling and Morphological Changes.

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Interleukin 23 (IL-23) stimulates survival and proliferation of Th17 cells that are implicated in autoimmune inflammatory diseases and IL-23 also serves as an important cytokine regulator for these diseases. IL-23 is reported to activate a complex consisting of the IL-12Rβ1 as well as the IL-23 receptor. Here we report, using RT-PCR on mRNA from human dermal microvascular endothelial cells (HDMEC), that these cells possess the IL-12Rβ1 receptor. We therefore studied to which extent IL-23 could activate endothelial cell signalling and the possible consequences
with regard to migration, spheroid sprouting and morphological changes. In HDMEC, IL-23 caused a transient increase in p-Akt and p-mTOR with maximal phosphorylation between 10 and 20 min. GTP-Rac was formed after 1 min and we found enhanced polymerizations of actin filaments as well as cell shape changes after 2 hrs. Time laps experiments with phase contrast microscopy also revealed cell shape changes induced by IL-23 when cells were followed for 6 hrs. We did not see enhanced migration with IL-23 or activation of angiogenesis in a mouse cornea assay. Microarray analysis revealed upregulation of mRNA coding for SMADs that are important regulators of transcription. IL-23 caused a considerable increase in spheroid sprouting of pig aorta endothelial cells (PAE) as measured in a collagen gel assay. IL-23 also stimulated phosphorylation of Akt near the perinuclear area as well as phosphorylations of JAK2, STAT3, STAT5 and Erk1/2. These data show that IL-23 activates endothelial cells, not as a classical angiogenic factor, but as a regulator of cell shape, transcription and possibly also a modulator of capillary wall integrity.

179/B126
Angiopoietin-1/Tie2 Signal Inhibits Inflammation through Expression of Krüppel-Like Factor 2.
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Angiopoietin-1 (Ang1) is a ligand for endothelium-specific receptor tyrosine kinase Tie-2. In adult vasculature, Ang1/Tie2 signaling is thought to regulate both maintenance of vascular quiescence and promotion of angiogenesis. However, it has been unknown how Tie2 signal regulates these distinct biological functions. Recently, we have reported that Ang1 assembles distinct Tie2 signaling complexes in the presence or absence of cell-cell junctions (Nat. Cell Biol. 2008). Ang1 bridges Tie2 at cell-cell contacts, resulting in trans-association of Tie2 in the presence of cell-cell contacts. In clear contrast, in the isolated cells, extracellular matrix (ECM)-bound Ang1 locates Tie2 to cell-substratum interface. Furthermore, Tie2 activated at cell-cell or cell-ECM contacts leads to preferential activation of Akt and Erk, respectively. In this study, we further performed DNA microarray analyses to identify the genes regulated by trans-associated Tie2 and ECM-anchored Tie2. Ang1 regulated distinct sets of genes in the presence or absence of cell-cell contacts. Interestingly, in the presence of cell-cell contacts, Ang1 up-regulated the genes involved in vascular stabilization, which include Krüppel-like factor 2 (KLF2), zinc finger protein 36, C3H type-like 2, connexin 40, delta-like1 and delta-like 4. We also revealed that Ang1 induces KLF2 expression through a phosphoinositide 3-kinase (PI3K)/Akt-dependent activation of myocyte enhancer factor 2 (MEF2), and that Ang1-induced KLF2 expression attenuates inflammation. Collectively, trans-associated Tie2 stimulates transcriptional activity of MEF2 through a PI3K/AKT pathway, thereby inducing KLF2 expression and contributing to the maintenance of vascular quiescence.

180/B127
The Roles of PML Nuclear Bodies in Endothelial Cell Physiology.
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Promyelocytic leukemia protein (PML) nuclear bodies (NBs) are dynamic sub-nuclear compartments that play roles in several cellular processes including transcriptional regulation, apoptosis, and DNA repair. We have previously shown that tumor necrosis factor alpha (TNFα) induces formation of PML NBs and promotes the expression of matrix metalloproteinase-10 (MMP-10) in human umbilical vein endothelial cells (HUVECs). We have also found that inflammatory cytokines, such as TNFα, interferon alpha (IFNα) and interferon gamma (IFNγ), are inhibitory to angiogenesis in HUVECs and human microvascular endothelial cells (HMVECs). In addition, knockdown of PML significantly compromises TNFα- and IFNa-mediated inhibition of tube formation and cell migration. We further demonstrate that TNFα and IFNα induce Stat1 expression in HUVECs, and that knockdown of Stat1 inhibits TNFα- and IFNα-induced PML mRNA accumulation. Chromatin immunoprecipitation (ChiP) assays indicated that Stat1 associates with PML promoter. These data suggests that Stat1 is a candidate transcription factor
involved in cytokines-induced PML expression. Finally, we show that integrin beta1 (ITGB1) was significantly up-regulated in PML-knockdown cells at both mRNA and protein levels and that TNFα, IFNα and IFNγ treatment decreased ITGB1 expression at both mRNA and protein levels. Based on these data, we hypothesize that PML negatively regulates tube formation in endothelial cells, in part by regulating cell migration through inhibition of ITGB1 expression. Taken together, our data supports a model in which PML NBs are important players that mediate TNFα and IFNα activity to control endothelial cell fate.

181/B128
Caveolae Mediate Protease-Selective Signaling by Protease-Activated Receptor-1.
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Protease-activated receptors (PARs) are a family of G protein-coupled receptors (GPCRs) that elicit cellular responses to extracellular proteases. The coagulant protease thrombin contributes to inflammatory responses associated with vascular injury and thrombotic disease, whereas the anti-coagulant protease activated protein C (APC) promotes cytoprotective and anti-inflammatory signaling. Moreover, both thrombin and APC signal through PAR1 but elicit opposite effects on endothelial barrier permeability. APC and thrombin also differentially regulate Rac and RhoA signaling, small GTPases that control endothelial barrier permeability. The mechanism by which two different proteases activate the same receptor to elicit distinct cellular responses is not known. Strikingly, pre-incubation of endothelial cells with APC ablates thrombin-stimulated signaling and increased endothelial barrier permeability. However, in cells stably expressing caveolin-1 siRNA, APC cytoprotective signaling was abrogated, whereas thrombin-induced cellular signaling remained intact, suggesting that receptor localization to caveolar microdomains is critical for protease-selective signaling. Our studies further indicate that APC-activated PAR1 is retained at the cell surface, while thrombin induces rapid receptor internalization through clathrin-coated pits and lysosomal degradation. Interestingly, APC pre-incubation does not affect thrombin-induced PAR1 internalization or degradation. These findings suggest that thrombin and APC activate distinct PAR1 subpopulations that utilize distinct processes for regulation of receptor signaling and trafficking. Towards understanding the mechanisms responsible for APC-induced signal regulation we examined the distribution of PAR1 and signaling effectors in caveolae by detergent-free sucrose density fractionation. PAR1 and signaling components of cytoprotective pathways were found largely in caveolin-1 enriched fractions, while inflammatory signaling mediators were detected mainly in heavy fractions. Collectively, these findings suggest that spatial and temporal distribution of PAR1 and signaling effectors contribute to the ability of different proteases to selectively signal through PAR1.

182/B129
Role of AKAP12 in Endothelial Cell Wound Healing In Vitro.
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AKAP12 (gravin), a 300kD multivalent A-kinase anchoring protein, is localized at the cell periphery and may participate in cell signaling events at that location. Based on previous studies linking AKAP12 to cell motility and cell spreading, the aim of this study was to determine if AKAP12 plays a role in endothelial cell wound healing. To test this hypothesis, mechanical scratch wounds were induced in confluent human umbilical vein endothelial cells (HUVEC) in 8-well plates, after which the wounded cultures were treated with different concentrations of antisense and missense oligonucleotides for 48 hours. The effect of oligonucleotide treatment on gravin expression was assessed by western blot. In addition, the pattern of AKAP12 expression in wounded HUVEC monolayers was examined using immunofluorescence microscopy and AKAP12 expression in active, sub-confluent cultures and quiescent, confluent cultures of HUVEC was compared by western blotting. Cultured HUVEC demonstrated a density dependent profile of AKAP12 expression. Low density, proliferating HUVEC cultures showed high AKAP12 expression whereas confluent contact inhibited cultures exhibited weak expression. Furthermore, the
expression of AKAP12 was up-regulated at the wound edge in wounded HUVEC monolayers. Finally, the level of gravin expression and the extent of wound closure after 48 was reduced in HUVEC treated with antisense oligonucleotides as compared to control cells treated with missense oligonucleotides. The pattern of expression in cultured HUVEC and the results of the antisense experiments conducted in this study suggest that AKAP12 may play role in cellular activities associated with endothelial cell wound healing.

183/B130
Apoptosis Induction in HUVECs by Acocostatin.
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Disintegrins are proteins isolated from viper snake venom and are capable of inducing apoptosis through competitive ligand-binding to integrins. We isolated a PIII disintegrin-like protein (Acocostatin) containing disintegrin and cysteine-rich regions from the copperhead snake Agkistrodon contortrix contortrix. Our hypothesis is that acocostatin will induce apoptosis in Human Umbilical Vein Endothelial Cells (HUVECs). Acocostatin was cloned into a pGEX-KG bacterial vector and the resultant recombinant protein was purified using glutathione sepharose beads. Apoptosis was detected with Annexin-V-FITC and nuclear fragmentation assays on HUVECs. Camptothecin, a known inducer of apoptosis, was the positive control, while glutathione-S-transferase (GST) treated cells served as the negative control. Groups of 100,000 cells were treated with 5 \( \mu \)M camptothecin, acocostatin, or GST for 24 hours. Another subset of cells was left untreated. Results were analyzed through flow cytometry and FACSCalibur software. The following results support our hypothesis: acocostatin induced 32.8% apoptosis, camptothecin 41.2%, GST 5.4%, and untreated cells exhibited 3.0% apoptosis. To test for nuclear fragmentation, 200,000 cells were grown on slides and treated with 2.5\( \mu \)M GST or acocostatin for 24 hours. One slide was left untreated. Cells were then fixed for 1 hour with 3% formaldehyde solution and stained with 1\( \mu \)M Hoechst stain for 15 minutes. When the slides were viewed at 100x under epifluorescent microscopy, the acocostatin treated cells demonstrated nuclear fragmentation, which was not observed in the other cells. Current experimentation is underway to determine which region of the acocostatin gene is responsible for the induction of apoptosis in HUVECs.

184/B131
Cell Phone Radiation Activates p38MAPK/Hsp27 Stress Pathway.
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Induction of biological effects by mobile phone radiation (RF-EMF) remains a controversial issue. We have earlier demonstrated that RF-EMF (900 MHz GSM signal) alters gene and protein expression and activates stress response (phosphorylation of p38MAPK and Hsp27) in human endothelial cell line EA.hy926 (Leszczynski et al., Differentiation 2002, 70:120-129; Leszczynski et al., Proteomics, 2004, 4:426-431; Nylund & Leszczynski, Proteomics 2004, 4:1359-1365; Nylund & Leszczynski, Proteomics 2006, 6:4769-4780). We have also shown that this radiation might alter protein expression in the skin of human volunteers (Karinen et al. BMC Genomics 2008, 9:77). In the present study we have further examined effects of RF-EMF (1800 MHz GSM signal) on stress response in EA.hy926 cells by determining effects on the expression of stress proteins and on the expression and phosphorylation (activity) of several kinases located upstream of Hsp27. Cells were exposed to RF-EMF at specific absorption rate (SAR) of 2.0 W/kg & 5.0 W/kg for 15, 30 and 60 minutes. After the exposure expression of stress proteins (Hsp27, Hsp40, Hsp60, Hsp70, Hsp90, Hsp110) and expression/activity of kinases (M KK3/6, p38MAPK, MAPKAPK2) were examined by western blot. Temperature of cells during the exposure was continuously monitored and it remained throughout the experiments at 37 ± 0.2 deg. C. from 3 to 5 experiments were performed for each protein, time-point and SAR. Statistical analysis was performed using Kruskal-Wallis test. Obtained results show that RF-EMF had no effect on the expression heat shock proteins (activity was not examined). Stress kinases MKK3/6 and
MAPKAPK2 appeared to be activated at 5.0 W/kg but not at 2.0 W/kg SAR. Expression but not activity of p38MAPK was also altered at 5.0 W/kg. In conclusion, the obtained results support a notion that RF-EMF might activate cellular stress response.

185/B132
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Arterial endothelial cells (ECs) actively respond to biochemical and biomechanical stimuli, and these responses regulate normal vascular function and the progression of atherosclerotic disease. The nesprins are large Klarsicht, ANC-1, Synex Homology (KASH) domain-containing proteins that directly link cytoskeletal elements to the nuclear envelope in mammalian cells. Nesprin-1 and -2 associate with the actin cytoskeleton, while nesprin-3 interacts with the intermediate filament network via plectin, a cytoskeletal cross-linker. Because the cytoskeleton modulates biochemical and biomechanical signal transduction in ECs, understanding the interdependence of nesprins and cytoskeletal elements is critical to elucidating the potential role of nesprins in EC signaling. To interrogate nesprin-cytoskeletal connections, we used a variety of techniques including pharmacological disruption of specific cytoskeletal elements, siRNA-mediated knockdown of nesprin-3 (siN3), and a dominant negative KASH protein plasmid (dnKASH) which displaces nesprins from the nuclear envelope. Disruption of filamentous actin using cytochalasin-D elicited a ~40% decrease in whole-cell nesprin-1 intensity while having no significant effect on nesprin-3 intensity. Intermediate filament disruption with acrylamide led to doubling in nesprin-3 intensity relative to untreated cells. Preliminary data suggests that transfection with the dnKASH plasmid increased the distance between the microtubule organizing center (MTOC) and the nucleus. Transfection with siN3 also increased MTOC-nucleus distance (compared to reagent alone). When HAECs were subjected to a steady shear stress of 15 dyne/cm² for 2 hrs, the MTOC polarized upstream of the nucleus, and this was unaffected by treatment with either dnKASH or siN3. These findings suggest that the cytoskeletal filament network in HAECs modulates nesprin expression and that KASH domain proteins regulate MTOC positioning relative to the nucleus.

186/B133
A Novel Role of Substance P as an Injury Messenger for BMSC Mobilization.
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Tissue injury may create a specific microenvironment for inducing the systemic participation of bone marrow stromal cells (BMSC) in the repair process. Here we show that Substance P (SP) is an early injury-inducible factor, regulated by wound size, and induces the CD29+ cell mobilization. This function of SP was proved in uninjured mice, rats, and rabbits by its intravenous injection. Those SP-mobilized CD29+ cells were characterized to be BMSC by binding of intravenously injected biotin-SP on BMSCs in vitro and their multipotent differentiation capacities In Vitro and ectopic bone marrow formation in vivo. Both SP injection and transfused SP-mobilized BMSCs accelerated wound healing, particularly, epithelial-entraftment of the transfused cells in the injured tissue. Finally, we showed that SP stimulated transmigration of human BMSCs, cell proliferation, Erk1/2 activation, and nuclear translocation of beta-catenin in vitro. This finding highlights a novel function of SP as a systemically acting messenger of injury and a mobilizer of BMSC to participate in wound healing. This study was supported by grants SC3120 given to Dr Y Son.
187/B134
Identification of a New Angiogenic Factor Secreted from Osteoblasts.
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In bone fracture repair process, cell-to-cell communication between osteoblasts and endothelial cells is considered as important event. Though its importance, cellular communicator between osteoblasts and endothelial cells is a little known. We focused on secretary molecules from osteoblasts might be involved in endothelial cell angiogenesis. To find secretary molecule specifically increased in osteoblasts with angiogenic activity, we compared activity of conditioned medium from undifferentiated mesenchymal stem cells and osteoblasts on endothelial cells angiogenic functions. As a result, we could observe that secretary molecules from osteoblasts showed significant increases in endothelial cells migration. To identify migration-inducing molecule, RP-HPLC and ESI LC-MS/MS analysis were conducted. Among the several candidate molecules, we selected protein C. Many intracellular functions of protein C have been known and there are some reports about protein C secretion to extracellular region. But, there is no report about extracellular function of protein C, especially related to function as a ligand. First, we checked chemotactic activity of protein C on endothelial cells. We could observe that protein C-induced endothelial cells migration in dose-dependent manner. Also, another property of angiogenesis, tube structure formation was induced by protein C treatment. These activities of protein C were mediated by FGFR-1 activation and its downstream signaling molecules activation. SU5402, FGFR antagonist, and siRNA for FGFR-1 abolished protein C-induced endothelial cell migration and activation of signaling molecules by protein C. Based on this study, identification of protein C as a new angiogenic factor for endothelial cell will be helpful for understanding of the communications between osteoblasts and endothelial cells and applications in bone-related disease such as bone fracture.

188/B135
The Role of LSECS in the Thrombocytopenia Caused by Liver Xenotransplantation.

There are over 17,000 people on the waiting list to receive a liver for transplantation. In order to bridge the gap between the number livers needed and the number of livers available for transplantation, sources of liver other than deceased human must be examined. The pig liver seems optimal, since pigs are easily raised, metabolically similar to people, are accurate in size, and are more ethically acceptable than non-human primates. We have observed that a pig’s liver perfused with human blood will bind and remove human platelets from circulation. Our goal is to identify what causes platelet binding and block the interaction. In this study porcine livers were perfused with human platelet rich plasma ex vivo. Following addition of PRP, The number of platelets in circulation decreased by over 90% at 15 minutes. Needle biopsies and samples of the perfusate were taken over 3 hours. Western blot analysis revealed that there was no sign of platelet activation or lysis during perfusion. Fluorescently labeled platelets were taken up by liver sinusoidal endothelial cells (LSEC) and compartmentalized in LAMP1 positive phagosomes based on confocal analysis. To confirm our ex vivo findings, we isolated primary LSECs from the pigs liver and found that they phagocytosed human platelets In Vitro independent of antibody binding and Fc receptor mediated phagocytosis. We have discovered that LSECs in the pig liver contribute significantly to the thrombocytopenia that occurs after xenotransplantation.

189/B136
Direct and Indirect Roles of Placental Macrophages in Early Vascular Development.
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Establishment of the placental circulation early in pregnancy is essential to allow the developing fetus access to maternal oxygen and nutrients. Placental vessels are believed to form in situ by the differentiation of endothelial precursors within the villous stroma; placental macrophages or Hofbauer cells (HCs) are abundant within stroma of early villi but their functions are not well understood. Here we use In Vitro cultures of primitive placental mesenchymal cells (PMCs) to test the hypothesis that HCs contribute to placental vascular development. HC in first trimester placental villi express CD163. Cultures of mixed PMCs generated from first trimester placenta (n=4) were separated into CD163+ (HC) and CD163- populations using immuno-magnetic beads. These were cultured separately or as a recombined population overnight on collagen I or MatrigelTM in the presence of EGF (10ng/ml) and FGF (20ng/ml) and monitored using time-lapse imaging. HCs were observed in close association with early placental vessels. CD163+ HCs cultured on MatrigelTM exhibited an elongated tubular morphology compared with those cultured on collagen I and became organised into primitive vascular structures (PVSs). Mixed CD163+/CD163- cultures formed significantly more PVSs than CD163- (HC depleted) cultures (p=0.014). Time-lapse imaging of mixed cultures on MatrigelTM revealed a close association of HCs with other stromal cells, and an apparent role for HCs in the co-ordination of network formation. These data support the hypothesis that these cells have the capacity both to stimulate and contribute to placental vasculogenesis and angiogenesis. Time-lapse investigations indicate that direct contact with PMCs may be important in the co-ordination of vascular patterning. Furthermore, our finding that HCs are capable of integrating into vascular-like networks In Vitro allows us to make the novel suggestion that they play a direct role in placental vessel formation through incorporation into vessels. Improved understanding of the mechanisms involved in placental vascular development will help identify therapeutic targets for diseases of pregnancy, such as Fetal Growth Restriction, in which vascular development is compromised.

190/B137
A Drosophila Model for Seamless Tube Formation.
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Objective: to identify the genetic and molecular mechanisms controlling seamless tube formation. The Drosophila tracheal system is composed of a network of tubes that sprout from 10 pairs of epithelial sacs. The network forms by a series of successive branching and fusion events. During this process, specialized tip cells lead the migration of new branches and mediate their interconnection. During the branch fusion process, some tip cells become specialized as “fusion cells” and convert themselves into seamless tubes that span the branch fusion joint. Other tip cells become specialized as “terminal cells” that go on to form branched, blind-ended seamless tubes that ramify extensively on target tissues, like muscle, and act as the site of gas exchange. Similar to tracheal terminal and fusion cells, endothelial tip cells that connect new sprouts to other tubes in the vascular network during angiogenesis have been shown to form seamless tubes. So-called “seamless endothelial cells” are believed to create seamless tubes by a cell hollowing process in which vesicles and vacuoles are trafficked to the center of the cell and coalesce and fuse to form an intracellular lumen bounded by apical membrane. A forward genetic screen for defects in seamless tracheal tubes has identified novel factors, such as whacked, that are involved in membrane trafficking. The primary defect in whacked mutants is the overgrowth of tubes at the distal tip of terminal branches. Positional cloning of whacked revealed that it encodes a RabGAP protein. Interestingly, overexpression of Whacked results in a striking phenotype, causing the overgrowth of tubes proximal to the terminal cell nucleus. Additional work has revealed that Rab35 is the key target of Whacked RabGAP activity: expression of a constitutively active isoform of Rab35 phenocopies whacked loss of function and expression of a dominant negative isoform of Rab35 (Rab35DN) phenocopies the Whacked overexpression defect. Moreover, expression of Rab35DN in the tracheal system can suppress whacked terminal cell morphogenesis defects and rescue whacked mutant animals to viability. Thus, we predict that Rab35, and its regulator Whacked, direct polarized tube growth.
Actin (191 – 203)

191/B138
Assembly Dynamics and Stabilization of Actin-Like Parm Filaments from Plasmid R1.
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In bacteria many low-copy plasmids are actively transported through the cytoplasm by the assembly of cytoskeletal polymers. In the case of Type II plasmid segregation the polymer is an actin-like protein, ParM, and the free energy of its assembly is harnessed by a DNA protein complex composed of the repressor protein, ParR, and the centromeric DNA sequence, parC. We investigated the assembly dynamics of ParM using Total Internal Reflection Fluorescence (TIRF) microscopy combined with stochastic modeling of polymer assembly. Modeling indicates that filaments must be stabilized by a cap of at least three ATP bound protomers at the end of the filament. Using TIRF microscopy of quantum dot-labeled ParR/parC with various combinations of wildtype and nonhydrolyzing ParM mutants we tested the hypothesis that the ParR/parC complex forms a sliding collar that fits around the ParM filament and that it tracks growing ends of ParM filaments by binding more tightly to newly incorporated, ATP-bound ParM subunits than to older subunits that have hydrolyzed ATP. This test was based on our prediction that the ParR/parC complex will bind tightly to clusters of non-hydrolyzing mutants and be displaced from the growing end of the filament.

192/B139
Origin of Twist-Bend Coupling in Actin Filaments.
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Actin filaments are semi-flexible polymers on cellular length scales that display large scale conformational twisting and bending motions. Modulation of filament bending and twisting elasticity has been linked to regulatory actin binding protein function, filament assembly dynamics, force generation and motility (work output). The coupling between bending and twisting modes is a critical determinant of overall polymer elasticity and mechanics, but has not been evaluated for actin filaments. We have adopted a coarse-grained description of an actin filament that captures key documented features of actin filaments including the protomer subunit structure, geometrical constraints coming from filament helicity and twist, interaction energies and bending and twisting elasticities. We have used this computational model to predict the degree of coupling between filament bending and twisting motions. Comparison with a "single-stranded" actin protofilament indicates that bend-twist coupling in actin originates from the double protofilament structure.

193/B140
Force Regulates Actin Subunit Interactions through Catch-Slip Bonds Switchable by Formin.
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It has been shown that cyclic uniaxial stretch induces the cell and its actin stress fiber to align in two ways: 1) with RhoA, and its effector, formin, the cell aligns perpendicular to the direction of stretch; 2) if RhoA or formin is inhibited, it aligns parallel to stretch. This suggests that the way actin cytoskeleton dynamics respond to force in-vivo is modulated by the RhoA/formin pathway. To pursue the molecular mechanism underlying this observation, we studied the bond lifetime-force relationship of G-actin/G-actin interactions by atomic force microscopy (AFM). Both AFM
probes, the cantilever tip and the polystyrene surface, were functionalized with G-actin by incubating sequentially with biotinylated BSA, streptavidin, and biotinylated G-actin. The working buffer for lifetime measurements is F-buffer. The measured binding was specific to G-actin/G-actin interactions as the binding frequency was significantly suppressed by not coating the cantilever tip with G-actins, by using G-buffer as working buffer, or by adding latrunculin a to prevent G-actin/G-actin interactions. Without formin, the bond lifetime of G-actin/G-actin interaction first increases then decreases as the force increases, with a maximum of 0.63 sec occurring at 10.5 pN, which is a "catch-slip" mechanism. This behavior can be explained by force-induced formation of new interactions between G-actins, a structural mechanism provided by steered molecular dynamics simulations. When the FH2-containing formin construct, mDia1(FH2-DAD) is involved, the catch-slip bonds are switched to slip bonds, in which the bond lifetime monotonically decreases with the increasing force. The slip bonds are further reversed back to catch-slip bonds by the DID domain-containing N-terminal construct of mDia1, which inhibits FH2 via DAD-DID interaction, an auto-inhibition which can be removed by RhoA. Our study suggests how actin dynamics can be regulated by force and how this regulation is modulated by formin and RhoA, thus potentially explain how the force-induced cell alignment is regulated by the RhoA/formin pathway. It indicates a possible crosstalk mechanism bridging the actin-mediated mechanotransduction and the biochemical signal transduction pathways.

194/B141
A Role for an Antiparallel Actin Dimer in Cells.
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In the cytoplasm, actin is a major component of the cytoskeleton and its coordinated assembly of filaments from monomers drives fundamental biological processes including cell motility, division and membrane dynamics. During the past decade, actin has been identified as an essential component of several nuclear machines that regulate gene expression and chromosome architecture. It may also contribute to the structural integrity of the nucleus, and mediate intranuclear movement of viruses, nuclear organelles, and even chromosomal sites. Despite the involvement in numerous nuclear mechanisms, the polymerization status of actin in the nucleus is still under debate. At the onset of actin polymerization in vitro, two types of dimers are detected. First, an antiparallel dimer ("lower dimer" or LD) occurs (1). As polymerization proceeds, LD decreases, while a parallel filament dimer ("upper dimer") appears. It has been proposed that the ragged and branched filaments observed during the early stages of polymerization might be attributed to a transitory LD incorporation (2). However, because of its transient nature, LD has so far evaded detection in vivo. Because a previous study demonstrated that antibodies are suitable tools to examine different forms of actin (3), we set out to raise LD-specific to study the functional significance of this actin conformation. Activity against monomeric actin was depleted from serum of rabbits immunized with crosslinked LD, resulting in a serum that was highly enriched for LD-reactivity. Immunoelectron microscopy of In Vitro polymerized actin filaments revealed specific labeling of filament branches. Moreover, for the first time the presence of LD in cells was demonstrated by immunofluorescence. The labeling pattern indicates that this unconventional form of actin is present in the cytoplasm and in the nucleus and is distinct from the actin forms described so far. Bibliography: 1.- Millonig R, Salvo H, Aebi U. J Cell Biol 1988 106:785-96 2.- Schoenenberger CA, Bischler N, Fahrenkrog B, Aebi U. FEBS Lett 2002 529:27-33 3.- Schroeder U, Graff A, Buchmeier S, Rigler P, Silvan U, Tropel D, Jockusch BM, Aebi U, Burkhard P, Schoenenberger CA. J Mol Biol 2009 386:1368-81

195/B142
Characterizing the Steric Effects of a Yeast/Muscle V76i Substitution in Actin.
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Actin inter-subdomain interactions are important for controlling actin conformational dynamics and consequently its ability to reversibly polymerize. Yet the intramolecular allosteric interactions responsible for conformational regulation are largely unknown. Studies of a series of yeast actins containing muscle residues in subdomains 1 and 2 showed that the replacement of V76 in yeast actin with I in muscle actin results in a substantial retardation of nucleotide exchange rate. This result coupled with the actin structure led us to hypothesize that hydrophobic interactions involving the residue at position 76 coordinate the interaction of subdomains 1 and 2 with the interdomain nucleotide cleft. We thus altered the degree of packing by constructing V76I, W79Y and W79F mutant yeast actins and V76I/W79Yor F double mutants to relieve the crowding introduced by I76. Of the single mutants, only W79Y showed abnormal growth characteristics which were rescued by the addition of I76. As with the hybrid actins, I76 alone resulted in a nucleotide exchange rate half that of WT actin. W79F in addition to I76 partially rescued this retardation. I76 slows nucleation and polymerization, but these effects are exacerbated, not rescued, in the double mutant. Although yeast profilin binds both V76I and the V76I/W79F double mutant with an affinity similar to that of WT actin, it only accelerates their nucleotide exchange rates to a level half of that achieved with WT actin. Thus, profilin cannot compensate for the steric effects imposed by the introduction of I76. Interestingly, profilin retards polymerization of these two mutant actins, especially V76I/W79F actin to a much greater extent than it does WT actin. Together, our results suggest that the extent of hydrophobic interactions provided by residue 76 plays a major role in regulating the interaction between subdomain 1 and 2 of actin and consequently its nucleotide exchange and polymerization properties.

196/B143

Cytoplasmic Actin Isoforms Perform Distinct Functions in Stereocilia.
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About 100 actin-rich stereocilia sprout from the top of auditory hair cells and are arranged like a choir in rows of increasing height with members of a given row nearly the same height. In addition to adopting a precise morphology, stereocilia must be maintained for life because hair cells are not regenerated. Stereocilia are largely made of β- and γ-actin, which are encoded by Actb and Actg1, respectively. These actin proteins are nearly identical, differing by only four biochemically-similar amino acids. Nevertheless, Actb and Actg1 knockout mice have distinct phenotypes. Actg1−/− mice developed progressive hearing loss corresponding with disruptions in stereocilia F-actin. In addition, individual stereocilia became shortened or lost while neighboring stereocilia appeared unaffected. (Belyantseva IA, Perrin BJ, Sonnemann KJ, et al. PNAS (2009) 106:9703). Hair cell-specific Actb knockout mice also developed progressive hearing loss, but did so more rapidly than Actg1−/− mice. Furthermore, β-actin-deficient stereocilia had a distinct phenotype. Most prominently, the first row of stereocilia in the hair cell bundle became dramatically shorter coincident with the onset of hearing loss. In addition, stereocilia within a row lost precise height registration with neighboring stereocilia. Together, these data are consistent with γ-actin strengthening stereocilia or repairing damage while β-actin maintains stereocilia length.

197/B144

Differential Arginylation of Actin Isoforms Is Regulated by a Novel Degradation Mechanism Coupled to the Translation and Folding Dynamics In Vivo.
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Actin cytoskeleton in non-muscle cells is composed of beta and gamma isoforms that differ only by four N-terminal amino acid residues, but perform distinct functions in vivo. One of the insights into the underlying mechanisms maintaining this functional distinction came with the recent discovery that beta, but not gamma actin can be posttranslationally arginylated on its N-terminus, and that this modification bears important regulatory role in actin dynamics and maintaining the integrity of the actin network. Here we addressed the question of how the preferential arginylation of only one of the highly similar actin isoforms can be achieved, by examining the metabolic fate of exogenously expressed arginylated and non-arginylated actin in non-muscle cells. We found that arginylated gamma actin is highly unstable In Vivo compared to the arginylated beta actin,
and that it degrades selectively by a proteasome-dependent mechanism. Remarkably, in addition to the difference in amino acid sequences, this instability is regulated at the nucleotide level at the translation level. Further investigation suggests that preferential degradation of gamma actin is coupled to its slower translation rate compared to beta, leading to a slower folding, that exposes a lysine residue normally hidden in the actin structure, for ubiquitination. Our data suggest a novel mechanism of protein regulation by degradation coupled with translation/folding dynamics, likely employed for the differential regulation of arginylation in vivo. Our data is the first demonstration that beta and gamma actin are synthesized with different rates and that these rates likely confer differences in their posttranslationally modified states, important for their in vivo functions.

198/B145
Ion-Dependent Polymerization Differences between β- and γ-Nonmuscle Actins.
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Eight different point mutations in mammalian γ-nonmuscle actin cause autosomal dominant deafness, and many of these mutations are in regions that may affect binding sites for proteins that regulate filament formation and stability. Hair cells contain β- and γ-nonmuscle actin and are one of the few cell types in which the γ isoform predominates over the β-form (2:1 ratio). The molecular mechanism(s) leading to this actin-dependent deafness phenotype is unknown. As a model system for assessing the biochemical effects of these mutations, we previously showed that five of the six mutations in yeast actin demonstrate near normal polymerization kinetics. This result led us to hypothesize that the deafness phenotype largely results from altered actin/actin-binding-protein interaction(s). Although yeast and γ-actins are 90% identical, the remaining difference may be important for assessing the effects of these mutations in humans. We thus established a baculovirus expression system to allow us to begin an initial characterization of human β- and γ-nonmuscle actin before studying the effects of the deafness mutations in the γ isoform. The two nonmuscle actins differ by only 4 amino acids, three of which are in the amino-terminus and none of which are near a polymerization interface. Negatively-stained samples of pure β- and γ-actins show essentially the same morphology. Surprisingly, β-nonmuscle actin in the Ca form polymerizes and releases phosphate over twice as fast as γ-nonmuscle actin. In the Mg form, these differences become much smaller. Mixing assays show the two actins co-polymerize and demonstrate that the difference in polymerization results largely from an elongated γ-actin nucleation phase. In the hair cell stereocilia, a phalloidin-staining core of bundled β-actin filaments is surrounded by a shell of γ-actin that does not stain with phalloidin. The stereocilium also contains about 1 mM calcium-binding proteins including parvalbumin which could provide an ionic environment favoring the Ca-actin form. Our polymerization results are consistent with the disposition of the two isoactins in the hair cell stereocilium.

199/B146
Identification of a Novel γ-Actin Transcript Enriched in Muscle.
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Cytoplasmic actins are among the most ubiquitous and abundant proteins in the nucleated cell. Most vertebrates express two isoforms of cytoplasmic actin, β and γ (ACTB and ACTG1, respectively), which differ by only four amino acids in the N-termini of the proteins. The coding sequence of these two isoforms is remarkably well conserved, whereas the introns and UTRs demonstrate significantly less conservation. Although much is known about their general function, tissue specific distribution and relative expression levels, distinctly different functional roles for these two isoforms have not yet been determined. In most tissues, β-actin is expressed at >2:1 ratio relative to γ-actin, with the exception of the cochlea and gut epithelia where γ-actin is the predominant isoform. Expression of ACTG1 is thought to be controlled in part by limiting the splicing of intron 3 from the RNA transcript. Using PCR we have identified a novel γ-actin
transcript that is present in mouse cDNA from muscle, brain, eye, intestine, and cochlea. This novel transcript is particularly enriched in muscle where it is present at a level nearly equal to that of the standard isoform. This transcript includes a novel 45 bp exon located in the middle of ACTG1 intron 3 that introduces a premature stop codon. This 45 bp segment is flanked by canonical splice sites and shows a high degree of conservation between species. Likewise, we have demonstrated the presence of this alternative transcript in mouse cDNA from humans, cats, and dogs. A protein product corresponding to either a truncated protein or read-through of the stop codon is not present in the tissues. We therefore postulate that ACTG1 regulation is not controlled by the splicing of intron 3 from the RNA transcript, but rather involves the inclusion of a stop codon-containing pseudoexon/alternative exon. Lloyd C, Gunning P. β- and γ-actin genes differ in their mechanisms of down-regulation during myogenesis. J Cell Biochem. 2002;84(2):335-42.

200/B147
Actin Conformational Changes Induced by Profilin Binding Observed with H/D Exchange and Mass Spectrometry.
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Profilin binds to actin’s barbed end across subdomains 1 and 3 leading to an increased rate of exchange of the adenine nucleotide bound in actin’s inter-domain cleft. However, the mechanism by which this increase is achieved is not understood. There is also an actin isoform-specific difference in actin’s ability to bind to different profilins leading to a differential enhancement of nucleotide exchange. Again, the mechanism underlying this functional specificity is not well understood. We used amide proton hydrogen/deuterium (HD) exchange detected by mass spectrometry to analyze conformational changes induced by profilin binding to yeast and muscle actins. Areas of yeast actin affected by binding of yeast profilin (YPF) or human profilin 1 (HPF) at saturating conditions are subdomain 1 peptides 8-31, 95-104 and 356-375, subdomain 4 peptide 208-218, and subdomain 3 peptide 154-176. All five peptides exhibit less exchange when YPF is bound compared to actin alone. On the other hand, YPF increases HD exchange in peptide 326-340 in the area connecting subdomains 1 and 3. A comparison of HD exchange of yeast actin bound to YPF or HPF shows differences in peptide 347-355 in subdomain 1. Furthermore, peptide 154-176 exchanges more when HPF rather than YPF is bound. Binding of HPF1 to muscle actin leads to an increased exchange over actin alone in peptide 145-155 in subdomain 3 and peptide 326-340 connecting subdomains 1 and 3. Conversely, HPF decreases exchange in peptide 208-218 in subdomain 4. Interestingly, binding of YPF to muscle actin causes greater HD exchange in the area connecting subdomains 1 and 3 than does HPF. The results demonstrate possible differential interaction of specific profilin isoforms to specific actin isoforms. They also show YPF-dependent propagation of conformational change through subdomains 1 and 3 onto subdomain 2 of yeast actin, while HPF seems to affect mostly subdomain 3 in muscle actin. Many of the changes observed are in peptides that line or contact the nucleotide cleft, consistent with profilin’s ability to alter the kinetics of nucleotide exchange.

201/B148
RAGE Regulation of F-Actin Remodeling and Membrane Repair/Permeability in Endothelial Cells.
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Receptor for Advanced Glycation End products (RAGE), an immunoglobulin superfamily transmembrane protein, is a receptor for multiple ligands. Ligands of RAGE include AGE (advanced glycation end product), s100, HMGB1, and b-amyloid (Ab). Thus, RAGE is implicated in the pathogenesis of multiple disorders, including diabetic complications, Alzheimer’s disease, and chronic inflammations. However, the underlying mechanism for RAGE induced cellular dysfunction remains largely unknown. Here, we present evidence for RAGE regulation of F-actin remodeling and membrane repair/permeability in endothelial cells. An endothelial cell line expressing RAGE showed a defective membrane repair activity and a decrease of F-actin stress
fibers and associated focal adhesions as compared with the parental cell line. The RAGE effects appeared to require serum in the culture and the intracellular domain of RAGE. These results suggest a new cellular function of RAGE in regulating plasma membrane permeability, an event that may underlie its pathogenesis of multiple disorders, including Alzheimer’s disease.

202/B149  
**Peritubular Myoid Cells Contract in Both the Parallel and Transverse Axes.**  
*L. Lopez, A. Losinno, A. Morales, J. Cavicchia; IHEM, UNCuyo, Mendoza, Argentina*

Testicular peritubular myoid cells (PMCs), the main cellular component of the seminiferous tubule (ST) wall, are contractile cells that express cytoskeletal markers of true smooth muscle, such as alpha-isoactin, F-actin, and myosin. The contractile activity of PMCs is responsible for the contraction of STs underlying the transport of spermatozoa and testicular fluid and, at least in part, for sperm release during spermiation. PMCs from isolated ST segments VII-VIII of rat testis, either relaxed (control ST) or contracted with endothelin-1 (E-1 ST) were analyzed by confocal and electron microscopy to visualize the actin filament cytoskeleton (AF). PMC areas from control and E-1 ST were 938 ± 64 and 760 ± 81 (µm² ± SEM) respectively and x, y diameters from control and E-1 ST were x: 36 ± 1; 30 ± 2 and y: 30 ± 2; 23 ± 3 (µm ± SEM) respectively. PMCs from these segments contain two layers of actin filaments. One layer is localized underneath the nucleus and close to the basal membrane of the seminiferous epithelium and is oriented transversally to the major ST axis. The other layer is localized above the nucleus and oriented parallel to the ST axis. Contraction reduced the area of the PMCs, and the x and y axes and also shortened both the parallel and transverse layer of AF. These results lead us to conclude that the particular organization of actin filaments in PMCs allow them to contract the cell in both parallel and transverse axes.

203/B150  
**Modulation of β Actin Filament Network Associated to Sertoli Cell Ectoplasmic Specializations along the Rat Seminiferous Epithelium Cycle.**  
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The term blood-testis barrier (BTB) originated by early physiological studies on the mammalian testis that demonstrated a differential transport of ions and metabolites between the basal areas of the seminiferous tubules (basal compartment), and the apical areas (adluminal one). Its morphological correlate was found to be located in specialized junctional devises found between the adjacent Sertoli (sustentacular) cells, integrated by occluding or tight junctions, which seal each inter-Sertoli space and by adherens junctions conformed by a highly organized circumferential belt of β actin filaments close to Sertoli cell membranes. Another junctional device, the tubular bulbar complexes (TB) completes this arrangement. These structures were termed ectoplasmic specializations (ES) by L. Russell(1977).OBJECTIVE: The aim of this study was to evaluate the distribution and arrangement of β actin and the implicated Sertoli membranes using confocal or electron microscopy, colocalization of beta actin with prosaposin (that specifically stains Sertoli cytoplasm) and freeze fracture (to observe Sertoli membranes and TB complexes).RESULTS: A clear, differential spatial organization of β actin filaments together with a differential arrangement of TB complexes along the spermatid cycle were observed. CONCLUSION: Significant interrelations between the actin network, the junctional complexes of the BTB and TB complexes were detected at different stages of the seminiferous cycle. Supported by Cuyo University and CONICET (National Research Council) grants from Argentina.

**Actin Associated Proteins I (204 – 217)**

204/B151  
**Analysis of Domain(s) of LASP-2 Responsible for Actin-Binding Activity and Localization in Filopodia and Focal Adhesions.**
Lasp-2 concentrates to lamellipodial actin bundles in neural cells and focal adhesions in fibroblastic cells. Lasp-2 has three structural regions: a LIM domain, a nebulin-repeat region, and an SH3 domain. Lasp-2 is a splicing variant from the LASP2/NEBL gene, which concurrently encodes nebulette, an actin-binding protein expressed specifically in cardiac muscle. Various nebulin repeats of nebulin superfamily (nebulin, nebulette, and N-RAP) have been shown to associate with F-actin, and it has been proposed that the repeats participate in the association of lasp-2 with F-actin; however, the domain(s) responsible for actin-binding activity and the localization are still unclear. To determine the region responsible for the actin-binding activity of the lasp-2 molecule, we prepared recombinant fragments of lasp-2 in order to subject them to a co-sedimentation assay with F-actin. We expressed the fragments examined in the co-sedimentation assay with F-actin in NG108-15 neuroblastoma cells to confirm the relationship between the actin-binding activity and subcellular localization of lasp-2. The fragments was also transfected in C2C12 myoblasts to analyze localization in focal adhesions visualized by interference reflection microscopy. We revealed that the domains responsible for actin-binding activity showed a similar subcellular localization to full-length lasp-2 in NG108-15. However, the domains did not primarily contribute to its recruitment to focal adhesions in C2C12. Actin filament binding activity may be a major contributor to the subcellular localization of lasp-2 to filopodia but is not crucial for lasp-2 recruitment to focal adhesions.

205/B152
Dissection of Functional Domains of GSNL-1, a Gelsolin-Like Protein in C. elegans.
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The Caenorhabditis elegans gelsolin-like protein-1, GSNL-1 (475aa), is a new member of the gelsolin family of actin-regulatory proteins (Klaavuniemi et al., 2008). It is an unconventional gelsolin-related protein with four gelsolin-like domains (G1 to G4), unlike typical gelsolin-like proteins with six or three G domains. Sequence alignment suggests that GSNL-1 lacks two G domains that are equivalent to fourth and fifth G domains of gelsolin. GSNL-1 severs actin filaments and caps the barbed end in a calcium-dependent manner similarly to gelsolin. In contrast, GSNL-1 has different properties from gelsolin in that it remains bound to F-actin, and does not nucleate actin polymerization. To understand the mechanism by which GSNL-1 regulates actin dynamics, we investigated domain-function relationship of GSNL-1 by analyzing activities of truncated forms of GSNL-1. For actin filament severing activity, G1 plus the linker between G1 and G2 was sufficient, whereas the presence of G2 greatly enhanced the activity. This is similar to gelsolin. Effects on the critical concentration of actin suggested that G1-G2 was necessary and sufficient for barbed-end capping activity. G-actin binding assays by non-denaturing electrophoresis demonstrated the presence of at least two G-actin binding sites in G1 and G3-G4. By contrast, F-actin co-sedimentation assays indicated that G3-G4 was sufficient for binding to F-actin, which is different from gelsolin that has an F-actin binding site in G2. Interaction of gelsolin with actin was inhibited by direct binding of gelsolin with polyphosphoinositides (PIP2). Actin severing activity of GSNL-1 was also inhibited in the presence of PIP2. Two PIP2-sensitive domains were mapped to G1 and G2. These results suggest that G domains of GSNL-1 play distinct roles in regulation of actin dynamics and that F-actin binding sites are located in different G domains between GSNL-1 and gelsolin.

206/B153
Kinetic Analysis of the Binding of Cofilin, 14-3-3y and AZX100.
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Pathological scarring, such as keloid and hypertrophic, is characterized by the presence of myofibroblasts, which are distinguished from fibroblasts by the expression of α Smooth Muscle
Actin (αSMA) and Connective Tissue Growth Factor (CTGF). Previous data have shown that AZX100, a 24 amino acid phosphopeptide analog of HSP20 linked to a protein transduction domain, can reduce the expression of αSMA and CTGF in keloid-derived myofibroblasts. AZX100 binds the intracellular scaffolding protein 14-3-3γ, which displaces bound cofilin and results in actin depolymerization and reversal of TGF-β1-induced myofibroblast differentiation. In this study, Surface Plasmon Resonance (SPR) and immunoprecipitation (IP) studies were used to determine whether AZX100 can displace bound cofilin from 14-3-3γ. The interaction of 14-3-3γ and AZX100 was examined using SPR. 14-3-3γ binds to AZX100 in a phosphorylation-dependent manner with two distinct KD’s of 642±40 nM and 803±51 nM. No binding of 14-3-3γ to unphosphorylated or scrambled AZX100 was observed. Similarly, binding of 14-3-3γ to cofilin was phosphorylation-specific, as no binding to unphosphorylated cofilin was observed. Analysis of the interaction between 14-3-3γ and cofilin revealed two distinct KD’s of 42±6 nM and 267±75 nM. Most importantly, the ability of AZX100 to displace bound 14-3-3γ from phosphorylated cofilin was demonstrated by SPR analysis as well as by immunoprecipitation experiments. These results demonstrate that although 14-3-3γ has a lower binding affinity for AZX100 versus cofilin, a molar excess of AZX100 can displace bound cofilin, leading to actin depolymerization in the myofibroblast. Thus, AZX100 may be of therapeutic benefit for the prevention of abnormal scarring.

207/B154
Overlapping and Distinct Functions for Cofilin, Coronin and Aip1 in Actin Dynamics In Vivo.
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Actin filament disassembly is critical for actin-based motility, to control filament network architecture and to regenerate subunits for assembly. Here, we examined the roles of three actin cytoskeletal proteins, coronin, cofilin and Aip1, which have been implicated as combining in various ways to control actin dynamics by promoting or regulating disassembly. We studied their functions during the endocytosis process in budding yeast, where actin filament dynamics at the cortical actin “patch” contribute to the formation and movement of endocytic vesicles. We found that all three proteins were recruited during the late phase of the actin patch life. They all arrived at the same time, when actin and other actin-associated proteins were leaving the patch. Their peak molecular stoichiometry per patch was 500 cofilin molecules, 90 coronin molecules and 45 Aip1 molecules. We found that mutations in genes encoding one protein could influence the proper localization of another protein. Using quantitative patch motion analysis and comparing mutant alleles, we found commonalities and differences in the phenotypes for mutations of the three genes. Together, the results are consistent with all three proteins working to promote actin disassembly, but not in a simple way and not with equal importance.

208/B155
All Three Vertebrate Iqgaps Interact with Actin Filaments.
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The IQGAP family comprises scaffolding proteins that regulate actin assembly, exocyst function, and cellular motility, morphogenesis, adhesion and division. Vertebrates express 3 family members, IQGAP1, IQGAP2 and IQGAP3, which are ~60-70% identical to each other in amino acid sequence and are organized into a common set of functional domains. Prior work has shown that IQGAP1 binds directly to F-actin (Bashour et al. 1997. J CELL BIOL 137: 1555-1566), and stimulates nucleation of branched actin filaments through N-WASP and the Arp2/3 complex following direct binding to cytoplasmic domains of ligand-activated growth factor receptors (Benseñor, et al. 2007. J CELL SCI 120: 658-669). IQGAP1 thus spatially and temporally couples growth factor signaling to actin assembly, and by extension, the plasma membrane expansion that defines cell migration and shape change. By contrast to IQGAP1, very little is known about
interactions between IQGAP2 or IQGAP3, and actin. To begin addressing this issue we used actin filament binding assays and fluorescent fusion protein expression in cultured mammalian cells. Purified his-tagged IQGAP2(full length) and IQGAP2(2-528), an N-terminal fragment corresponding to ~1/3 of the full length protein, bound directly to purified F-actin by high speed centrifugation assays. Furthermore, IQGAP2(full length)-YFP and IQGAP3(full length)-YFP, as well as the N-terminal fragments, IQGAP2(1-528)-YFP and IQGAP3(1-531)-YFP, all targeted to F-actin rich domains, including lamellipodia and cell-cell junctions, when expressed in mammalian cells. Similar observations were made for IQGAP1(full length)-YFP, IQGAP1(1-522)-YFP and GFP-IQGAP1(1-550). With 2 exceptions, IQGAP2(full length)-YFP and IQGAP2(1-528)-YFP, none of the fluorescent fusion proteins associated with stress fibers. Fluorescent fusion proteins of the middle and C-terminal thirds of each IQGAP failed to target to actin filaments. Based on these observations, we hypothesize that all 3 mammalian IQGAPs interact directly with actin filaments in vivo via their N-terminal regions, and that the unique localization of IQGAP2 on stress fibers signifies functional heterogeneity among the IQGAPs. Supported by NIH grant NS051746 to GSB.

Distinct Functional and Structural Interactions between Iqgap1 and Cdc42 Versus Rac1.
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IQGAP1 stimulates nucleation of branched actin filaments by activating N-WASP, which in turn activates the Arp2/3 complex (Benseñor, et al. 2007. J CELL SCI 120: 658-669). N-WASP can be activated by other factors, including GTP-bound forms of Cdc42 and Rac1, which also bind IQGAP1. We report here the use of purified proteins for In Vitro polymerization and binding assays, and of FRET microscopy of cultured cells to illuminate functional interactions involving IQGAP1, N-WASP, and either Cdc42 or Rac1. Pyrene-actin assembly assays were used to compare how activated Cdc42 and Rac1, alone and in combination with IQGAP1, affected actin assembly in the presence of N-WASP and the Arp2/3 complex. Cdc42 (100-200 nM) and IQGAP1 (45 nM) cooperatively stimulated actin filament nucleation, primarily by reducing the lag time before Vmax for assembly was reached. This nucleation cooperativity reflected dose-dependent stimulation by Cdc42 of IQGAP1 binding to N-WASP. Under otherwise identical conditions, Rac1 and IQGAP1 behaved quite differently. at low (100 nM) Rac1, the two proteins cooperatively reduced the lag time before assembly Vmax was reached, but at high (200 nM) Rac1 Vmax was faster and was reached more quickly for Rac1 alone than for either IQGAP1 alone, or the combination of Rac1 and IQGAP1. This negative cooperativity reflected dose-dependent inhibition by Rac1 of IQGAP1 binding to N-WASP. These results imply that IQGAP1 interacts by distinct mechanisms with Cdc42 versus Rac1 to fine tune actin filament assembly in vivo. To address this possibility, quantitative FRET microscopy fully corrected for spectral bleedthrough was used to study interactions of GFP-IQGAP1 with mOrange-Cdc42 versus mOrange-Rac1 in live MDCK cells. Robust corrected energy transfer was observed for both FRET pairs, but the average distance between IQGAP1 and Cdc42 was 0.7 nm with a range of 0.65-0.77 nm, whereas IQGAP1 and Rac1 were 0.74 nm apart on average, with a range of 0.70-0.80 nm. The differential interactions of IQGAP1 with Cdc42 versus Rac1 that we observed In Vitro were thus recapitulated in live cells, and both sets of data support a higher affinity of IQGAP1 for Cdc42 as compared to Rac1. Supported by NIH grant NS051746 to GSB.

Roles of Ena/VASP and Capping Protein in Drosophila Development.
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Proper development requires cells to build a range of different actin-based structures. Each actin structure presumably arises from differing underlying actin geometries resulting from the effect of different suites and levels of actin regulators. We use Drosophila morphogenesis and oogenesis as models for understanding In Vivo requirement of proper actin regulation, specifically focusing on two integral actin regulators: Enabled/VASP (Ena) and Capping Protein (CP). In oogenesis we found Ena plays important roles in cortical integrity of germ-line nurse cells, and in proper formation of bundled actin filaments in nurse cells during the dumping process. Ena localizes to barbed ends of these specialized actin structures. Ena appears to be negatively regulated by Abelson kinase in this context. Analysis of ena mutants affecting different domains suggests the EVH1 domain is not as critical as the oligomerization and EVH2 domains in oogenesis. Further insight into the contribution and importance of individual domains of Ena during morphogenesis and oogenesis is currently being pursued through a protein structure function approach. We found CP is important for nurse cell dumping, and also plays a surprisingly critical role in oocyte specification perhaps as part of the dynactin complex. Together these data support an antagonistic relationship between Ena and CP in oogenesis. We are continuing to explore the relationship between these two proteins in morphogenesis.

211/B158
**Actin Polymerization in Differentiated Vascular Smooth Muscle Cells Requires Vasodilator-Stimulated Phosphoprotein (VASP).**

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Our group has previously shown that vasoconstrictors increase net actin polymerization in differentiated vascular smooth muscle cells (dVSMC) and that increased actin polymerization is linked to contractility of vascular tissue. However, the underlying mechanisms are largely unknown. Here, we evaluated the possible functions of the Ena/VASP family of actin filament elongation factors in dVSMC. Inhibition of actin filament elongation by cytochalasin-D decreases contractility without changing the level of myosin light chain phosphorylation, suggesting that actin filament elongation is necessary for dVSM contraction. VASP is the only Ena/VASP protein highly expressed in aorta tissues and VASP knockdown decreased smooth muscle contractility. VASP partially colocalizes with both alpha-actinin and vinculin in dVSMC. Profilin, known to associate with G-actin and VASP, also colocalizes with alpha-actinin and vinculin, potentially identifying both the dense bodies and the adhesion plaques as hot spots of actin polymerization. Differential centrifugation and imaging results indicate that VASP may undergo subtle positional changes in response to stimuli. The EVH1 domain of Ena/VASP is known to target these proteins to their sites of action. Introduction of an expressed EVH1 domain as a dominant negative inhibits stimulus-induced increases in actin polymerization. We also directly visualized, for the first time, the incorporation of rhodamine-labeled actin in dVSMC cells, and identified “hot spots” of actin polymerization in the cell cortex that colocalize with VASP. These results indicate a role for VASP in actin filament assembly at the cell cortex that modulates contractility in dVSMC.

212/B159
**Remodeling of the Actin Cytoskeleton by Vasodilator-Stimulated Phosphoprotein Regulates the Formation of Dendritic Spines and Synaptic Strength.**

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Reorganization of the actin cytoskeleton is critical for the formation and plasticity of dendritic spines, which are actin-rich structures that constitute the postsynaptic sites of excitatory synapses. Alterations in the density and morphology of spines, which can be attributed to actin remodeling, are associated with neuronal disorders, including mental retardation, Fragile-X syndrome, Down’s syndrome, Alzheimer’s disease, and epilepsy. This points to a central role for these structures and the regulation of actin in cognitive function. Despite the importance, the
mechanisms that regulate actin reorganization in dendritic spines are currently not well understood. Here, we show that the multifunctional actin-binding protein vasodilator-stimulated phosphoprotein (VASP) is enriched in spines and functional synapses in hippocampal neurons. Knockdown of endogenous VASP using siRNA leads to a significant decrease in the number of spines and synapses. Expression of GFP-VASP results in an increase in the number of spines and the size of spine heads with a higher concentration of filamentous actin and more available barbed ends in these structures. In addition, VASP promotes an increase in the synaptic expression of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)-type glutamate receptors at the membrane, suggesting that VASP strengthens synaptic efficacy. Expression of VASP lacking the Ena/VASP homology domain2 (EVH2), which binds G- and F-actin, results in a reduction in synaptic targeting, spine density, and spine head size. Deletion of the coiled-coil domain, which mediates VASP tetramerization, within EVH2 decreases the number and size of spines. Interestingly, the EVH1 domain, but not the proline-rich region, is also important for VASP function in spines. Collectively, our results suggest that actin polymerization and bundling, which are mediated by VASP, regulate the density, size, and morphology of dendritic spines. Therefore, VASP-regulated remodeling of the actin cytoskeleton is critical for the formation and expansion of dendritic spines and for modulating synaptic strength.

213/B160  
The Role of Cyclase-Associated Protein 1 (CAP1) in Actin Dynamics.  
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The actin cytoskeleton is essential for many cellular processes such as morphogenesis, motility, endocytosis and cytokinesis. The structure and dynamics of the actin cytoskeleton are regulated by various actin binding proteins. Cyclase-associated proteins (CAPs) are highly conserved actin monomer binding proteins found in all eukaryotes studied. Two CAP isoforms are found in mammalian cells, CAP1 is widely expressed in non-muscle cells and CAP2 is found mainly in developing and mature striated muscles. Budding yeast has only one CAP protein (called Srv2), which binds ADP-actin monomers and profilin through its C-terminal domain, and cofilin/ADP-G-actin complex through its N-terminal domain. By mutagenesis analysis, we show here that mouse CAP1 binds ADP-G-actin, profilin and cofilin/ADP-G-actin by similar mechanisms to yeast Srv2. However, unlike yeast Srv2, mouse CAP1 binds also ATP-G-actin with high affinity through its WH2 domain. We are also currently carrying out RNAi and rescue experiments with mutant versions of CAP1 to reveal the roles of these various protein-protein interactions in morphogenesis and motility of mammalian cells. In conclusion, here we show that mammalian CAP1 binds profilin, ADP-G-actin, ATP-G-actin and cofilin/actin through distinct sites.

214/B161  
CAP2, Cyclase Associated Protein 2, Is a Dual Compartment Protein with WH2 Domains.  
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CAPs are evolutionary conserved proteins that have roles in regulating the actin cytoskeleton and in signal transduction. Mammals have two CAP genes that code for related proteins, CAP1 and CAP2. With specific antibodies for both proteins we studied their distribution and subcellular localisation. CAP1 shows a broad tissue distribution, whereas CAP2 is significantly expressed only in brain, heart and skeletal muscle, and in skin. CAP2 is found in the nucleus in undifferentiated myoblasts and at the M-line of differentiated myotubes. In PAM212, a mouse
keratinocyte cell line, CAP2 is enriched in the nucleus. By contrast, CAP1 localises to stress fibers and F-actin rich regions such as lamellipodia in PAM212 cells. In human skin CAP2 is present in all living layers of the epidermis where it localises to the nuclei and to cell-cell junctions. ChIP analysis revealed that CAP2 could effectively bind to chromatin. Moreover, like other CAPs, CAP2 can sequester actin through its C-terminal domain. This sequestering activity presumably resides in the WASP homology domain 2 (WH2 domain), a short stretch of amino acids between the N- and C-terminal domain of CAP2. Immunofluorescence studies with mouse whole mount embryos revealed the expression of CAP2 at E12.5, specifically in the developing heart. Thus CAP2 is an important actin binding protein, which sequesters actin through its WH2 domain, with a dual compartmental distribution and may play an important role during heart development in mouse embryogenesis.

**215/B162**

**Selective Disruption of the Actin Cytoskeleton but Not the Muscle Sarcomere by Targeting Cytoskeletal Tropomyosin.**

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The actin cytoskeleton represents a promising target for the design of cancer chemotherapeutics, but to date no anti-actin compounds have reached the clinic. Directly targeting actin is associated with unacceptable toxicity due to the central role of actin in striated muscle contraction. To maintain cardiac and respiratory function, compounds which disrupt actin filaments must discriminate between the actin cytoskeleton of tumor cells and the specialized actin-based structures of the striated muscle sarcomere. Muscle and non-muscle actin filaments are associated with distinct tropomyosin (Tm) isoforms which regulate filament function. Our laboratory has designed novel anti-Tm compounds which specifically target non-muscle Tms relied upon by cancer cells. OBJECTIVE: to investigate the specificity of anti-Tm compounds by assessing their impact on cardiac function In Vitro and In Vivo. SUMMARY OF RESULTS: The structural integrity of isolated rat cardiomyocytes (CMs) was maintained with exposure to the lead anti-Tm compound, TR100, at a concentration (10 μM) which disrupted adhesion and viability of the neuroblastoma tumor cell line, SKN-Be2C. To assess function following TR100 treatment, sarcomere shortening of contracting isolated adult rat CMs was measured in real time using the IonOptix system. Acute perfusion with 10μM or 50μM TR100 did not alter the contractile properties of the CM, measured as peak shortening, duration of contraction and maximal rate of shortening and relengthening. In contrast, all contractile parameters were significantly depressed with exposure to the actin destabilizing compound, Cytochalasin-D (20μM). In Vivo cardiac data was derived from a B16/F10 murine melanoma model, treated with 30mg/kg TR100. at this dose, tumor growth was suppressed, but no obvious signs of hypertrophy or heart damage were observed as measured by changes to interventricular septum thickness, heart weight or blood troponin I levels. CONCLUSIONS: These results demonstrate that it is possible to target the actin cytoskeleton to achieve tumor-specific cytotoxicity without consequent cardiac toxicity.

**216/B163**

**Tropomyosin 4 Affects Neuronal Morphogenesis and Differentiation.**

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The actin cytoskeleton regulates cellular structure and a range of functions such as cell motility, cytokinesis, and vesicle formation. Tropomyosins regulate these functions by attenuating effects of other actin binding proteins on actin filaments. at least 40 Tropomyosin isoforms are produced
from four genes (αTm, βTm, γTm, and δTm). Tm4 is the single known mammalian product of the δTm gene, with similar distributions along membrane junctions in many tissues, including synapses. Tm4 function in brain is largely unknown. We have identified a potential alternative product from the δTm gene, which is immunoreactive with the Tm4 antibody. We aim to identify and characterise this product, and to investigate the function of Tm4 in neural cells. Methods Rat neuroblastoma cells (B35s) were stably transfected with a mammalian vector containing Tm4 cDNA. We are using proteomics approaches to identify which proteins are affected in their expression by Tm4 overexpression. We have optimized the purification of the Tm4 antibody immunoreactive product, and are currently preparing samples to sequence this product. Results Endogenous and exogenous Tm4 associates with actin cables in B35s, and Tm4 overexpression induces morphological changes normally associated with differentiation. The alternative novel product of Tm4 has been immunoprecipitated along with Tm4 and is resistant to heat treatment, indicating it may be a Tropomyosin. Conclusion Overexpression of Tm4 profoundly alters the actin cytoskeleton. We are currently investigating the molecular pathways through which Tm4 induces these changes. Identification of a potentially novel Tropomyosin isoform will be followed by characterization of the function of this isoform.

217/B164

Binding of Human Angiogenin Inhibits Actin Polymerization.
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Angiogenin is a potent inducer of angiogenesis, a process of blood vessel formation. It interacts with endothelial and other cells and elicits a wide range of cellular responses including migration, proliferation, and tube formation. One important target of angiogenin is cell-surface actin that has been shown to act as a receptor for angiogenin in endothelial cells and their interaction might be one of essential steps in angiogenin-induced neovascularization. We used pyrene-actin fluorescence, co-precipitation and electron microscopy to study the binding interactions between angiogenin and actin in a wide range of conditions. We showed that at physiological KCl concentrations, angiogenin inhibits polymerization of G-actin. At low KCl concentrations angiogenin induces formation of unstructured aggregates, which, as shown by NMR, may be caused by angiogenin’s propensity to form dimers and higher oligomers in solution. Angiogenin binds to F-actin. This binding does not cause depolymerization of actin filaments though it causes straightening of filaments. We tested if the presence of tropomyosin, an actin filament binding protein, can prevent angiogenin binding and vice versa. Binding of tropomyosin and angiogenin to F-actin appears to be independent therefore these proteins do not share the same binding interface on F-actin. These observations suggest that angiogenin binding to G-actin as well as to F-actin may cause drastic changes in the cell cytoskeleton by inhibiting polymerization of G-actin and changing the mechanical properties of F-actin.

Conventional Myosin (218 – 234)

218/B165

Myosin-II as an Active Force Sensor That Converts Mechanical Inputs into Chemical Signals.
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Mechanosensing is important in many physiological processes such as hearing and blood pressure regulation. However, the mechanisms by which cells sense mechanical inputs are not
well understood. Previous studies from our lab showed that cellular mechanosensing is crucial for regulating cytokinesis shape change. We use micropipette aspiration (MPA) to generate stress on the cell cortex. Using this assay, we discovered that mechanical stress stimulates the accumulation of myosin-II (a contractile force generating protein) and cortexillin-I (an actin bundling protein) to the deformation site to correct cell shape. Both myosin-II and cortexillin-I are required for this active mechanosensory system during cytokinesis. Recently, we demonstrated that this mechanosensory response is tunable by varying the lever arm length of myosin-II heavy chain, showing that myosin-II is an active force sensor in this mechanosensory system. In our new study, we focus on how mechanical inputs mediated through myosin-II lead to changes in biochemical signaling pathways, specifically the cortexillin-I regulatory and spindle signaling pathways. Rac1A (a small GTPase), IQGAP1, and IQGAPA (GTPase effectors) can form complexes with cortexillin-I. In the absence of both IQGAP1 and IQGAPA, cortexillin-I does not localize normally to the cleavage furrow during cell division. Using MPA, we found that IQGAPA, but not IQGAP1, is essential for myosin-II mechanosensing during cell division. Consistently, only IQGAPA is responsive to mechanical perturbation. Kif12, a mitotic-kinesin-like protein in Dictyostelium cells, is part of the chromosomal passenger complex, including INCENP and Aurora kinase. Kif12 is also recruited to the cell cortex inside the micropipette in a myosin-II-dependent manner. Thus, some of the mitotic spindle signaling machinery is responsive to the mechanical stress sensed by myosin-II. Overall, these results indicate that myosin-II is a key element for mechanosensing and for translating mechanical inputs into biochemical signaling pathways.

219/B166
Analysis of Conformation of the Skeletal Muscle Myosin Modified by Bifunctional Reagents Using FRET.
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Previously biochemical studies have demonstrated that the highly reactive cysteine residues SH1 and SH2 can be crosslinked by variety of bifunctional reagents with different spans (3-14 Å) in the presence of nucleotides, suggesting that the region is highly flexible. The SH1-SH2 region is believed to play a key role in the conformational changes that occur in the myosin head during the force generation coupled to ATP hydrolysis. We have previously shown that the HMM, which SH1-SH2 was crosslinked by p-Phenylenedimaleimide (pPDM) and 1,5-difluoro-2,4-dinitrobenzene (F2DNB) in the presence of ADP, have a novel conformation using quick freeze deep etch electron microscopy (QFDE-EM). We have also demonstrated that conformational change of the myosin motor domain during ATP hydrolysis can be monitored by measuring the FRET using fluorescent ATP analogue NBD-ATP. In the present study, we analyzed the conformation of the myosin crosslinked by pPDM and F2DNB using FRET between the ATP binding site and the A1 essential light chain (ELC). We prepared skeletal muscle myosin subfragment-1 (S1), which ELC was labeled by 6-bromoacetyl-2-dimethylaminonaphthalene (BD) at the Cys 177. And fluorescent ADP analogue NBD-ADP was trapped within the ATPase site of S1 labeled by BD. The FRET efficiency was estimated by measuring the change of fluorescence intensity of BD comparing with control BD-S1. The FRET efficiency of pPDM-S1-ADP and F2DNB-S1-NBD-ADP were lower than S1-NBD-ADP state. This suggests that the pPDM-S1-ADP and F2DNB-S1-ADP states form more kinked conformation

220/B167
Tropomyosin and Myosin-II Cellular Levels Promote Actomyosin Interactions and Contractile Ring Assembly in Fission Yeast.
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The actin-based myosin-II motor (Myo2p) and the actin-binding protein tropomyosin (Cdc8p) are essential for contractile ring formation and cytokinesis in fission yeast. Yet how these proteins
operate in the contractile ring is not well understood. We used a combination of genetics, live cell imaging, and biochemical assays to understand the roles of Myo2p and Cdc8p in ring assembly. We found that rings form prematurely in cells engineered to carry an extra copy of myo2 suggesting that changes in Myo2p cellular levels can influence ring assembly. Mutations in the Myo2p motor or Cdc8p delay ring formation, indicating a role for both proteins in ring assembly. Doubling Myo2p levels suppressed ring assembly and cytokinesis defects associated with a cdc8 mutation suggesting that Cdc8p contributes to effective Myo2p function in the cell. In Vitro assays revealed that Cdc8p regulates Myo2p directly. Actin-activated ATPase assays showed that association of Cdc8p with actin increases Myo2p's ATP hydrolysis rate by promoting its affinity for actin. In the presence of Cdc8p, Myo2p's ability to support actin filament gliding in motility assays was enhanced, despite a two-fold reduction in velocity. We found that Cdc8p promotes actomyosin interactions by favoring the strong actin-bound state of Myo2p. This new mode of regulation relies on the ability of tropomyosin to selectively alter myosin kinetics to maximize actomyosin interactions, which favors contractile ring assembly in the fission yeast system.

221/B168
Characterization of the Effects of Streptozotocin-Induced Diabetes on Expression of Myosin-LIB in Rat Brains Using Calmodulin Affinity Chromatography.
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The Ca+2/calmodulin complex interacts with and regulates various enzymes and target proteins, known as calmodulin-binding proteins (CaMBPs). This group of proteins includes molecular motors such as myosins. Myosin-II binds calmodulin in a Ca+2-independent manner and was showed in our study to be overexpressed in the brains of diabetic rats. In this study, we compared the calmodulin affinity chromatography-purified CaMBPs isolated from the brains of streptozotocin-induced diabetic rats and non-diabetic rats. Proteins eluted with EGTA and urea were separated on an SDS-PAGE gel, digested and submitted to peptide mass fingerprinting. The gel revealed similar protein profiles for the CaMBPs from the brains of diabetic rats and non-diabetic rats. There were sixteen intense bands for the diabetic brain and fifteen for the non-diabetic brain. Four proteins were found exclusively in diabetic brains, two were found exclusively in non-diabetic brains and thirteen were found in both types of rat brains. Moreover, the fraction eluted with 6 M urea contained myosin-IIB and cytoplasmic actin and showed that these proteins were strongly expressed in diabetic brains. Protein sequences were then analyzed for the presence of the calmodulin-binding sites. A large fraction of the proteins present (83%) had putative IQ motifs or calmodulin-binding sites. This is the first study to identify calmodulin-binding proteins in the brains of diabetic and non-diabetic rats through a comparative proteomic study, and it opens up new areas of study related to the link between myosin-IIB levels in the brain and diabetes mellitus.

222/B169
Conditional Ablation of Nonmuscle Myosin II-B in Mice Results in Arrhythmogenic Right Ventricular Cardiomyopathy Associated with Defects in the Coronary Circulation.
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Global ablation of nonmuscle Myosin II-B (NM II-B; B¹⁻¹ B¹⁺⁻) in mice results in lethality during embryonic development with cardiac associated defects. In an effort to produce a mouse model for human cardiac disease, we generated a NM II-B conditionally ablated mouse in which Cre-recombinase is under the control of the transgelin/SM22α promoter (B¹⁺⁻SM22α B¹⁻⁻SM22α mice). NM II-B was ablated in almost all cardiac myocytes, the majority of epicardial cells, and smooth muscle cells of the aorta as early as E11.5. Unlike B¹⁻⁻ B¹⁺⁻ mice, B¹⁺⁻SM22α B¹⁻⁻SM22α mice survive up to 5 months of age. MRI analyses of the mouse hearts show severe dysfunction and dilation of the right ventricle at 4 months, while the contractile activity of the left ventricle is not impaired.
EKG analyses of B<sup>SM22<sub>a</sub></sup> mice at 1 month and later show cardiac arrhythmias, including defects in conduction such as atrial-ventricular dissociation and the presence of numerous premature ventricular beats indicating a blockage in cardiac conduction. Sectioning of B<sup>SM22<sub>a</sub></sup> hearts at 3 months demonstrates marked cardiac myocyte hypertrophy and interstitial fibrosis in the right ventricle. Cardiac myocyte hypertrophy is also observed in the left ventricle, but no obvious interstitial fibrosis is seen. In addition, B<sup>SM22<sub>a</sub></sup> hearts show defects in coronary vessel development during the early embryonic stages. Large size coronary arteries are missing in the right ventricle of adult B<sup>SM22<sub>a</sub></sup> hearts. Many of these mice die suddenly between 3-5 months of age due to cardiac arrhythmias. In addition both the systemic and pulmonary blood pressure of B<sup>SM22<sub>a</sub></sup> mice were significantly lower compared to their control littermates consistent with the idea that NM II-B in vascular smooth muscle is contributing to maintain the vascular tone.

223/B170
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Cell migration of human oral keratinocytes is a highly regulated and coordinated process that is dependent upon dynamic changes in the actin cytoskeleton. In a previous study, we showed that virulence factors secreted by Candida albicans targets the keratinocyte actin cytoskeleton and stimulates cell migration by ~2-fold. In this study, we examined the dynamic distribution of non-muscle myo2A and B during stimulated keratinocyte migration to determine their roles in this process. We used human oral keratinocytes (OKF6/TERT-2) co-cultured with hyphae-forming wild-type C. albicans. Rates of keratinocyte migration and the cellular redistribution of myo2A and B were determined at 0, 3 and 6 hours by multi-mode high resolution microscopy and quantitative image analysis. First we observed that the protrusive activity at the leading edge, analyzed kymographically, was significantly greater in C. albicans treated cultures than in untreated cultures. In addition, cell migration increased and the distribution of myo2A and B changed. Myo2A, which was localized throughout the cytoplasm in untreated cells, localized to newly formed actin stress fibers and focal adhesions (FAs) after 3 h of treatment. Myo2A was excluded from the leading edge of the lamellipodia that formed after 6 h of treatment. In contrast, Myo2B, detectable at the rear and the center of untreated cells, was mostly absent from actin stress fibers and FAs that formed at 3 h, but localized at the leading edge of newly formed lamellipodia at 6 h. Fluorescence intensity profiles illustrated both the coordinated redistribution of myo2 isoforms and their colocalization with the actin cytoskeleton. Cell migration and the localization of myo2 a and B were not affected with the C. albicans mutant SAP4-6Δ/SAP4-6Δ and non-pathogenic Saccharomyces cerevisiae. We conclude that infection with C. albicans activated cell migration through the simultaneous reorganization of the actin cytoskeleton and the redistribution of myo2A and B, findings that differs from published results with other cell types. C. albicans, a pathogenic fungus that infects the human epithelium may stimulate changes in the actin cytoskeleton as part of its invasion strategies.

224/B171
Non-Muscle Myosin II Regulation via Inhibitory Site Phosphorylation.
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Non-Muscle Myosin II (NM-II) interacts with filamentous actin to produce force. This acto-myosin cytoskeleton is involved in many different cellular processes, including cytokinesis and migration. A "monomer" of NM-II consists of two myosin heavy chains (MHC), two essential light chains (ELC) and two regulatory light chains (RLC). A vast number of studies have investigated the NM-II regulation via phosphorylation of the RLC at residues T18 and S19. This phosphorylation leads to NM-II activation and assembly. However, more recent work has uncovered putative "inhibitory"
sites on both the RLC (S1, S2, and T9) and MHC IIA (S1917 and S1944). In vitro studies have suggested that phosphorylation at these sites shift the equilibrium of NM-II towards the disassembled, inactive state. Our current studies utilize phosho-specific antibodies and GFP-tagged NM-II constructs containing phospho-mimetic and non-phosphorylatable point mutations to investigate the mechanism by which these sites are phosphorylated in live cells and the effect that this phosphorylation has on NM-II localization and function.

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Residues in the Hydrophobic Seam of the Myosin S2/LMM Hinge Impart Myosin Rod- and Muscle-Specific Properties.

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The subfragment 2/light meromyosin "hinge" region of Myosin II rods forms a less stable coiled-coil than flanking regions. Different hinge sequences may contribute to muscle specific properties. Transgenic replacement of a portion of fast muscle myosin hinge a (encoded by exon 15a) in Drosophila melanogaster indirect flight muscle (IFM) with slow muscle hinge B (exon 15b) increases rod coiled-coil propensity, rod and sarcomere lengths and decreases flight performance. Using confocal microscopy we confirmed an ~8% sarcomere length increase in hinge B-substituted IFM myofibrils. To characterize the hinges' nano-mechanical properties, we determined persistence length (PL) differences via electron microscopy and molecular dynamic (MD) simulations. Rotary shadowed 15b myosin molecules showed an ~22% increase in PL relative to 15a (64.2 vs. 50.3 nm) while MD simulations revealed an ~39% greater PL for 15b relative to 15a (85 vs. 52 nm). These data are consistent with a high coiled-coil propensity of exon 15b-containing rods potentially stiffening the hinge and a substantial portion of the tail. To determine hinge residues critical for imparting muscle, myofibrillar and molecular properties, we transgenically replaced either 2 charged (line 15aCh) or 2 polar (line 15apH) amino acids of 15a with uncharged or with hydrophobic residues, respectively, from 15b. PCOILS structure-prediction software estimates a low (8%) coiled-coil propensity for both 15a and 15aCh-expressing molecules. However, the coiled-coil propensity for 15apH molecules is predicted to be 60%, nearly the value for 15b-containing hinges (82%). Flight indices (4.1 of a possible 6.0) and myosin rod contour lengths (149.8 nm) for 15aCh were similar to 15a control flies, while 15apH flies showed dramatically compromised flight ability (1.8 of a possible 6.0) and expressed longer myosin rods (154.1 nm) as with 15b. We are determining sarcomere lengths from 15aCh and 15apH myofibrils and PLs of the myosin tails to quantify the stiffness of the hinges/rods. Our data suggest certain hydrophobic residues are critical for establishing rigid coil-coils and, in turn, hinge-specific properties that dramatically influence molecular and muscle-specific properties.

226/B173

Effects of the Alternatively Spliced Surface Loops on the Kinetics of NMHC-2C.

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Nonmuscle myosins are widely distributed and play important roles in the maintenance of cell morphology and cytokinesis. Until now, little is known about the function of NMHC-2C and its three splice variants. Here, we present the detailed kinetic and functional characterization of the three alternatively spliced isoforms of human nonmuscle myosin-2C. NMHC-2C is alternatively spliced both in loop-1 and loop-2. An 8 aminoacid (aa) insert in the loop-1 region is present in isoforms 1 and 3. Isoform 3 additionally displays a 33 aa insert in the loop-2 region. Isoform 2 contains no inserts in either of the loops and represents the shortest isoform. Baculovirus expression of the gene constructs encoding the motor domains of the NMHC-2C isoforms fused to an artificial lever arm results in the production of soluble and functional protein. Transient kinetic experiments indicate a more than 100-fold decrease in the affinity of isoform 3 for F-actin in the absence of nucleotide, which can be attributed to the loop-2 extension. ADP binding shows only minor differences for the three splice variants. In contrast, larger differences are observed for
the rates of ADP release both in the absence and presence of actin. The largest differences are observed between isoforms 2 and 3. Isoform 3 displays a 5-fold increase in $K_D$ and 12-fold increase in $K_{AD}$. Isoform 1 shows intermittent behavior. Additionally, our results indicate that the ADP release kinetics of all three isoforms are modulated by physiological changes in the concentration of free Mg$^{2+}$-ions. This work defines the functional properties and regulatory mechanisms for the NMHC-2C isoforms.

227/B174

Novel Mechanism of S100A4 Inhibition: Trifluoperazine-Induced Protein Oligomerization.

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S100A4, a member of the S100 family of Ca2+-binding proteins, regulates carcinoma cell motility via interactions with myosin-IIA. Numerous studies indicate that S100A4 is not simply a marker for metastatic disease, but rather has a direct role in metastatic progression. These observations suggest that S100A4 is an excellent target for therapeutic intervention. Using a novel biosensor-based assay, trifluoperazine (TFP) was identified in a screen for small molecule inhibitors as a compound that disrupts the S100A4/myosin-IIA interaction. To examine the interaction of S100A4 with TFP, we determined the 2.3 Å crystal structure of human Ca2+-S100A4 bound to TFP. Two TFP molecules bind within the hydrophobic target binding pocket of Ca2+-S100A4 in solution. Remarkably, TFP binding results in the assembly of ten Ca2+-S100A4/TFP dimers into a tightly packed pentameric ring. Within each pentamer most of the contacts between S100A4 dimers occurs through the TFP moieties. Equilibrium sedimentation and cross-linking studies demonstrate the formation of a similarly sized S100A4/TFP oligomer in solution, and an examination of the Hill coefficient indicates that TFP-mediated S100A4 oligomerization is cooperative. Assays examining the ability of TFP to block S100A4-mediated disassembly of myosin-IIA filaments, demonstrate that significant inhibition of S100A4 function occurs only at TFP concentrations that promote S100A4 oligomerization. These studies support a novel mode of inhibition in which TFP disrupts the S100A4/myosin-IIA interaction by sequestering S100A4 via small molecule-induced oligomerization.

228/B175

Regulation of Lamellipodial Persistence by S100A4 during Macrophage Chemotaxis.

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S100A4, a member of the S100 family of Ca2+-binding proteins, is directly involved in tumor metastasis. In addition to its expression in tumor cells, S100A4 is expressed in normal cells and tissues, including fibroblasts and cells of the immune system. To examine the contribution of S100A4 to normal physiology, we created S100A4-deficient mice by gene targeting. Homozygous S100A4-/- mice are fertile, grow normally and exhibit no overt abnormalities; however, bone marrow macrophages (BMMs) derived from these mice display significant defects in chemotactic motility. S100A4-/- BMMs exhibit a random walking path as compared to wild-type macrophages due to altered protrusive activity and a failure to form stable lamellipodia. Biochemical studies indicate that these changes are the consequence of myosin-IIA overassembly and associated perturbations on adhesion complexes. The loss of S100A4 expression also reduced the invasive capability of macrophages and did not support macrophage-dependent invasion of tumor cells into a collagen gel. These studies establish S100A4 as a regulator of physiological macrophage motility, and suggest a role for S100A4 in regulating macrophage recruitment and chemotaxis in vivo.
**229/B176**  
*Myosin-Specific Chaperone UNC-45 Is Required for Drosophila Cardiac Function and Myocardium Integrity.*  
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Using Drosophila melanogaster as a model organism we are defining the role of UNC-45, a putative myosin chaperone, in maintaining striated muscle function and integrity. UNC-45 belongs to the UCS (UNC-45, CRO1, and She4p) domain protein family, whose members interact with various myosins and are required for myosin function. The In Vivo RNAi approach using the Gal4/UAS system permits us to knock down Drosophila unc-45 (dunc-45) transcripts in a tissue-specific manner. DUNC-45 knock-down in the heart allows survival to adulthood, but lifespan is dramatically reduced, with ~90% dead within three weeks. Optical heartbeat analysis of semi-intact hearts from various age adults was carried out using high-speed video and movement analysis algorithms. Analysis of several cardiac parameters (e.g., diastolic and systolic diameters, heart period and arrythmicity index) showed severely compromised cardiac function. For example ~80% of 1, 2 and 3 week old knock-down hearts do not show contraction or relaxation of normally highly-contractile regions (primarily in abdominal segment three). The remainder of the heart shows a greatly reduced and irregular beating pattern. Another striking response to DUNC-45 knock-down was dilation of the third segment of the heart. For example, the average diastolic diameter for one week old control fly heart is 76 µm. However, in the 1 week old knock-down heart the average diastolic diameter is 128 µm. Cardiac arrhythmias were observed in one-week-old flies, whereas arrhythmias were only observed after three weeks of age in wild type flies. Immunofluorescent images of relaxed hearts using myosin antibody showed irregular heart muscle patterning and missing myofibrils in DUNC-45 knock-down adults. Structural and functional defects of the dunc-45 knock-down heart were rescued to some extent by transgenic over-expression of DUNC-45. As UNC-45 prevents folding and accumulation of myosin, the heart displays myofibrillar disarray and reduced contraction, which may account for the observed premature lethality.

**230/B177**  
*Structure of the Putative Myosin Chaperone, UNC-45.*  
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UNC-45 is a chaperone that may be responsible for folding the myosin motor domain into its correct conformation. Mutations in UNC-45 cause muscle defects and dysfunction, due to the importance of myosin in muscle structure and contraction. UNC-45 is composed of an N-terminal tetra-tricopeptide (TPR) domain, a C-terminal UCS (UNC-45/Cro1/She4 homology) domain, and a central region that links these. As Drosophila melanogaster is a model organism for muscle research, we are using it to investigate UNC-45’s role in muscle development and function. Here we present a 3.0 Å resolution x-ray crystal structure of Drosophila UNC-45 (dUNC-45), which should serve as a basis for attaining a detailed understanding of its mechanism of action. Bacterially expressed recombinant His-tagged dUNC-45 was purified sequentially using immobilized metal affinity chromatography and size exclusion chromatography. The protein eluted as a single peak, indicating a homogeneous population of protein suitable for crystallization. Crystals were prepared by hanging drop vapor diffusion and x-ray diffraction data were collected to a limit of 3.0 Å resolution. For phase determination, a seleno-methionine derivative dUNC-45 crystal was prepared for single wavelength anomalous dispersion (SAD) experiments. Synchrotron data were collected at the Berkeley National Laboratory Advanced Light Source. The diffraction data were processed in HKL2000. Selenium positions were determined and refined in Phenix. The resulting structure was refined against native data using Phaser and maximum-likelihood refinement with Refmac5. Model building was carried out in
The current model of dUNC-45 has $R$-cryst and $R$-free values of 0.24 and 0.28 respectively. The TPR domain is not visible in the model, likely due to flexibility of the domain within the crystal. The protein consists of multiple $\alpha$-helixes that form specific hydrophobic surface patches which could be involved in substrate binding. Currently, we are pursuing structure-based biochemical assays to determine the precise protein surfaces that are involved in the binding and chaperone activities of dUNC-45.

231/B178

**Cellular Characterization of the Last of the Dictyostelium Myosin II Heavy Chain Kinases - MHCK-D.**

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Contractile processes such as cytokinesis and cell migration rely on the proper assembly and localization of Myosin II bipolar filaments. In *Dictyostelium discoideum*, as well as in mammalian cells, Myosin II filament disassembly can be driven by phosphorylation of the Myosin II heavy chain (MHC) “tail”, preventing myosin-mediated contraction. In *Dictyostelium*, MHC phosphorylation is catalyzed by at least three kinases (MHCK-A, -B, and -C) that share homologous $\alpha$-kinase catalytic and WD-repeat domains. Another *Dictyostelium* protein, tentatively named MHCK-D, is predicted to have the same domain organization as the aforementioned MHCKs but its function in the cell is unclear. Previous studies showed that disrupting MHCK-D expression has little effect on Myosin II filament turnover in vegetative *Dictyostelium* cells. To examine further the role of MHCK-D in the regulation of Myosin II, we generated *Dictyostelium* cell lines over-expressing an amino-terminal truncation of MHCK-D that possesses both the kinase catalytic and WD-repeat domains (MHCK-D** cells). We found that MHCK-D** cells exhibited slowed growth in suspension culture and became large and multinucleated over time, with an average of 2.9 nuclei/cell after 5 days, compared with 1.2 nuclei/cell for AX2 cells grown under the same conditions. Analysis of cells expressing this same truncation of MHCK-D, but with a GFP tag, revealed a diffuse distribution of the kinase in the cell. RT-PCR studies indicate that MHCK-D is expressed constitutively throughout the developmental cycle of *Dictyostelium*. In summary, the Myosin II-null phenotype associated with MHCK-D over-expression suggests that this kinase can function as a MHCK that catalyzes MHC phosphorylation and drive Myosin II filament disassembly in the cell. We have also generated bacterial and *Dictyostelium* cell lines for the expression and purification of fusion-tagged MHCK-D for biochemical studies of the kinase’s activity.

232/B179

**Characterization of a Novel Myosin Regulatory Light Chain in Mice.**

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Previously, we systematically identified a number of novel, authentic genes abundantly transcribed in mouse cardiac muscle through in silico and In Vitro analyses. In the present study, we characterized one of such genes, Mm.261329. The Mm.261329 gene, currently named myosin light chain regulatory B-like (Mylrbl), is located on mouse chromosome 17, composed of 4 exons and transcribed into 1779-nucleotide mRNA encoding 172 amino acids. During the course of our study, we found the presence of a gene highly homologous to the Mm.261329/Mylrbl gene. This gene, designated as myosin light chain 12B (Myl12B), is adjacent to the Mm.261329/Mylrbl gene on the same chromosome. The Myl12B gene transcribes into 939-nucleotide mRNA encoding 172 amino acids of which sequence is remarkably similar (98%) to that of the Mm.261329/Mylrbl gene. Since none of these genes have been investigated at the protein and functional levels, we analyzed the biochemical and cellular characteristics of these proteins. The Mm.261329/Mylrbl protein was found to contain a Ca$^{2+}$ binding activity. We generated an antibody recognizing both the Mm.261329/Mylrbl and Myl12B proteins. Tissue distribution analysis showed that these proteins are expressed in diverse tissues including cardiac, smooth and non-muscle tissues. Proteomic analysis of proteins immunoprecipitated with the antibody in mouse heart revealed that interacting proteins are various myosin components different from
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those involved in muscle contraction. Immunostaining analysis of NIH3T3 cells showed that the Mm.261329/Mylrbl and Myl12B proteins are co-localized with the phalloidin-staining actin cytoskeleton. Thus, our results suggest implication of the Mm.261329/Mylrbl and Myl12B proteins in diverse cellular processes other than muscle contraction.

233/B180
Thrombin Regulation of Myosin Light Chain Phosphorylation in Retinal Pigment Epithelial Cells.
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The retinal pigment epithelium (RPE) forms a monolayer of quiescent cells that acts as an outer blood-retina barrier (BRB) between the choriocapillaries and the photoreceptors. An early event in the establishment of most retinal diseases involves BRB breakdown, whereupon serum components, thrombin among them, directly contact RPE cells. The serine protease thrombin activates cell signaling via G protein-coupled proteinase-activated receptors (PARs). Responses triggered by PAR stimulation include the activation of cytoskeletal targets which regulate cell shape and adhesion by inducing the formation of actin stress fibers, focal adhesion complexes and acto-myosin contraction. Thrombin-induced changes on the actin cytoskeleton could further compromise the barrier function of RPE cells, exacerbating the disease process. In order to support a role for thrombin in RPE cell cytoskeleton reorganization we analyzed its effect on the signal transduction pathways leading to myosin light chain (MLC) phosphorylation and actin polymerization in rat RPE cells in primary culture. The phosphorylation state of the MLC was determined by western blot analysis, changes in the RPE cytoskeleton were visualized using immunofluorescent staining for F-actin, and the activation of Rho GTPase was measured using an enzyme-linked immunosorbent assay. Our results show that thrombin induces the time-dependent phosphorylation of the MLC through the specific activation of PAR-1. Pharmacological analysis showed that this effect is mediated by at least four intracellular pathways: Rho/Rho kinase, protein kinase C (PKCζ), phosphatidylinositol-3 kinase/PKCζ and MLC kinase. Moreover, thrombin promoted actin stress fiber formation, which was significantly prevented by blockage of the pathways leading to MLC phosphorylation. Based on these results, we conclude that cellular contraction triggered by the increase in MLC phosphorylation, together with actin stress fiber formation, may be involved in RPE barrier dysfunction. This work was partially supported by Grant IN203507 from PAPIIT, U.N.A.M., and by Grant CB-80398 from CONACyT to A.M.L.C.

234/B181
Mouse Models of Human MYH9-Related Diseases.
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Humans with point mutations in MYH9, the gene encoding nonmuscle myosin heavy chain IIA (NMHC IIA), develop a variety of syndromes with defects in their platelets (macrothrombocytopenia), kidneys (glomerulonephritis), eyes (cataracts), hearing (deafness) and granulocytes (inclusion bodies). The purpose of the present study is to gain insight into the pathological mechanism of the diseases by generating mouse models for two of these mutations and characterizing the resultant mouse phenotypes. We have produced both R702C and D1424N mutant mice by using homologous recombination to replace wild type NMHC IIA with mutant R702C or D1424N in NMHC IIA. Breeding of heterozygous R702C mutant mice has not produced homozygous mutant offspring. The homozygotes die between embryonic day 8.5 and 10.5. on the other hand, breeding of heterozygous D1424N mutant mice produced homozygous mutant offspring at close to normal ratios, suggesting that mutations in the motor domain of NMHC IIA may have a more severe effect than mutations in the rod during embryonic development. Interestingly, giant platelets were found in the blood smears from both R702C and D1424N adult heterozygous mice. Both mutants also have significantly higher mean platelet volumes compared to their wild type littermates (6.12 +/- 0.62 fl, wt; 10.17 +/- 1.50 fl, heterozygous R702C; 10.13 +/- 1.66 fl, heterozygous D1424N). Kidney function was studied by examining the
albumin/creatinine ratio in urine samples. Some but not all adult heterozygotes of both mutant lines have higher albumin/creatinine ratios at 8-9 weeks, indicating that kidney impairment may develop in some heterozygous mutants at an early age. These preliminary results suggest that these mouse models should be useful in understanding the pathophysiology of human MYH9-related diseases.

**Tubulin (235 – 250)**

235/B182

**Tubulin Interaction with G Protein-Coupled Receptors Directs Receptor Transport from the Endoplasmic Reticulum to the Cell Surface.**

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The C-termini of G protein-coupled receptors (GPCRs) are required for their export from the endoplasmic reticulum (ER) to the cell surface. However the molecular mechanisms remain poorly understood. To address this issue, we searched for proteins interacting with the C-termini of GPCRs. By using peptide-conjugated Agarose affinity columns combined with proteomics, we identified α- and β-tubulin interacting with the C-terminus of α2B-adrenergic receptor (α2B-AR). The interaction was confirmed by GST-fusion protein pull down assays using purified tubulin and brain cytosolic extracts. Site-directed mutagenesis of the C-terminus identified the basic residue cluster in the membrane proximal amphipathic α-helix 8 as the tubulin docking site. This basic residue cluster is structurally similar to the tubulin-binding sites in the microtubule-associated proteins such as cytoplasmic linker protein-170, the end binding protein-1, CLIP170, XMAP215 and EB1. Interestingly, the positively charged Arg/Lys residues are highly conserved in the membrane proximal regions of the C-termini amongst family a GPCRs and indeed, tubulin interacted with the C-terminal positively charged motif in angiotensin II type I receptor (AT1R). Furthermore, mutation of the Arg/Lys clusters dramatically inhibited α2B-AR and AT1R transport to the cell surface and receptor-mediated ERK1/2 activation, and the mutated receptors were extensively arrested in the ER. These data provide the first evidence indicating that the basic residue clusters direct GPCR interaction with microtubules which coordinates receptor traffic from the ER to the cell surface (GM076167).

236/B183

**Characterization of Plant γ-Tubulin: Unique Amino Acid Residues and Function.**

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Plant microtubules are organized without obvious converging centers, while animal and fungal microtubules are mostly organized from focused organizing centers. How these plant-specific microtubule organization systems became established during plant evolution remains unknown. We and other groups have shown that γ-tubulin, a conserved microtubule nucleator protein, localizes to the microtubule nucleation sites in plant cells. We also have reported that the function of plant γ-tubulin is somewhat different from that of fungus (and presumably of animals) (Horio and Oakley, 2003, Plant Physiol.). We investigated the origin of plant-specific microtubule organization in terms of changes in amino acid residues and those in functions of various plant γ-tubulins. Among the plant lineage, gymnosperms are especially interesting, because only a primitive group of gymnosperms produce centrioles in their life cycle. Comparison of amino acid
residues of γ-tubulins showed that gymnosperm γ-tubulins were more similar to those of bryophytes/pteridophytes than to those of angiosperms irrespective of whether the plants have centrioles or not. These data clearly showed that γ-tubulins of land plants fall into two groups, angiosperm and non-angiosperm types. Next, we tested whether γ-tubulins of Ginkgo (non-angiosperm type) and of Chara (green algae) can replace the endogenous γ-tubulin in the fission yeast Schizosaccharomyces pombe, because severe defects occur when an Arabidopsis γ-tubulin (angiosperm type) is expressed in S. pombe. A strain expressing Ginkgo γ-tubulin grew much better at low temperatures and exhibited less morphological abnormality. A strain expressing Chara γ-tubulin showed even fewer defects. These results suggest that green plant γ-tubulins have gradually lost functions that are important for the cell growth of S. pombe, and, presumably, they were replaced by specific functions required for plant microtubule organization. This work was supported by JSPS grant 17207006 and SENTAN, JST.

237/B184
O-GlcNAc Modification on Tubulin Is Involved in Neurite Outgrowth of Dopaminergic Neuronal Cells.
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β-O-linked N-acetylglucosamine (O-GlcNAc) is a nucleocytoplasmic post-translational modification on serine and threonine residues that is dynamically regulated by O-GlcNAc transferase and O-GlcNAcase. Many proteins are O-GlcNAcylated in response to various cellular processes, including transcription, proliferation, apoptosis and signal transduction. In the case of neuronal cells, there are many O-GlcNAcylated proteins that are related to neurodegenerative diseases. Neuronal differentiation process is largely studied, but it is rarely known the relationship between O-GlcNAcylation and neuronal differentiation. To examine whether O-GlcNAc modification is involved in neuronal cell differentiation process, we utilized neurite outgrowth model system induced by all trans retinoic acid (tRA) in dopaminergic neuronal cell line. Total O-GlcNAcylation patterns were changed during tRA-induced neuronal cell differentiation. In addition, O-GlcNAc modification on neuron-specific tubulin beta 3 was decreased during neurite outgrowth. We confirmed O-GlcNAcylated peptide of tubulin alpha and beta using ESI Q-TOF. Also, increased O-GlcNAcylation of tubulin inhibits its heterodimerization and polymerization. Therefore, O-GlcNAcase inhibitors are co-treated with tRA to prevent the decrement of intracellular O-GlcNAcylation level, and the extent of neurite outgrowth was decrease 17% compared to tRA-treated neurons. Thus, our data indicate that O-GlcNAc modification on tubulin seems to be related to neurite outgrowth in cultured dopaminergic neuronal cells.

238/B185
Identification of a Novel Posttranslational Modification of Neuronal Tubulin by Transglutaminase and Polyamines.
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Neurons contain large numbers of microtubules which support intracellular transport, facilitate axon growth, and form the structural basis of neuronal morphology. While microtubules in non-neuronal cells are generally quite dynamic and can be depolymerized by cold, calcium, or antimitotic drugs, a significant portion of neuronal microtubules, particularly axonal microtubules, are resistant to such treatments and are unusually stable. Though the biochemical basis for such stability is still not clear, isoelectric focusing suggests that neuronal cold-stable microtubules contain biochemically distinct tubulins that are more basic in nature. Posttranslational addition of polyamines is one of the few posttranslational modifications that add positive charges to protein. Polyamines (putrescein, spermine and spermidine) are abundant cations in brain and transglutaminase can modify proteins by incorporating polyamines onto select glutamine residues via gamma-glutamyl amine covalent bonds. To test the hypothesis that transglutaminase-catalyzed polyamination of tubulins may contribute to microtubule stability, endogenous
polyamine levels were lowered in rats with DFMO, an irreversible inhibitor of polyamine synthesis. Significant decreases in neuronal cold-stable tubulin levels were observed. In Vivo labeling of tubulin with 3H-putrescine and an In Vitro transamidation assay using the fluorescent polyamine analogue monodansylcadaverine indicated that neuronal tubulin is a transglutaminase substrate and can be polyaminated. Further, the modification sites were mapped using LC-MS-MS which suggested a sequence specific incorporation of polyamines on neuronal tubulins in the presence of transglutaminase activity. Additional experiments demonstrated that endogenous brain transglutaminase-catalyzed polyaminated tubulins mimic neuronal stable microtubules regarding their resistance to cold and calcium, and 2D-PAGE of polyaminated tubulin showed a basic shift for modified tubulin. Together, these experiments suggested that axonal tubulin may be polyaminated upon activation of transglutaminase, and that this modification may contribute to the stability of axonal microtubules.

239/B186
A Hypothesis on the Origin and Evolution of Tubulin.
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Tubulin, the protein subunit of microtubules, is an α/β heterodimer. Other isoforms of eukaryotic tubulin include γ, δ, ε, η, and ζ. All these proteins are related to the eubacterial protein FtsZ, which is involved in cytokinesis, so the genes that encode all these proteins probably evolved from a common ancestor in the forebear of these two branches of current life. Of these proteins, only α, β and γ are known to participate in microtubule formation, although η and δ may bind to microtubule minus ends. Thus, the property of forming microtubules occurs in only one part of the tubulin family tree. Hence, a tubular assembly geometry is unlikely to have characterized the tubulin/FtsZ ancestor. FtsZ, α, β, and γ can all polymerize to form curving filaments or rings. Filaments of FtsZ are thought to change their curvature to constrict the bacterial membrane at the cell equator, and γ rings help to nucleate microtubules. α/β heterodimers form curved filaments whenever a microtubule is depolymerizing, and the same proteins can form rings during microtubule depolymerization in the presence of either high Mg2+ or certain drugs. Curving protofilaments of α and β tubulin appear to be connected via fibrils to the inner kinetochore, so their changes in curvature may help microtubule depolymerization to pull chromosomes toward the poles of a eukaryotic spindle during mitosis. In the case of FtsZ and α/β tubulin, GTP binding and hydrolysis appear to play a role in protein structure changes and thus in curvature changes. In short, it is likely that the common ancestor of the tubulin superfamily and FtsZ was a protein that polymerized to form curving filaments that exerted intracellular forces by a mechanism involving GTP.

240/B187
Myc-Nick Is Cytoplasmic Form of Myc That Promotes Tubulin Acetylation and Accelerates Muscle Cell Differentiation.
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The oncoprotein Myc is a basic HLH transcription factor essential for embryonic development, and a critical regulator of major biological processes such as proliferation, growth, apoptosis, and differentiation. Myc controls these processes by regulating specific target genes and promoting global chromatin changes. While transcriptional regulation of Myc target genes is being extensively studied, potential transcription-independent functions of Myc are just beginning to be explored and may explain some of the biological effects induced by Myc. In the process of investigating non-nuclear functions of Myc, we discovered Myc-nick, a cytoplasmic form of Myc that contains an intact N-terminus but lacks the C-terminal region (nuclear localization signal, Max dimerization domain and DNA binding domain). Myc-nick is generated by a proteolytic cleavage of full-length Myc that is carried out in the cytoplasm by calcium dependant calpains. Ectopic expression of Myc-nick in myc-null fibroblasts caused major changes in the cytoskeleton and promoted dramatic changes in cell morphology marked by the induction of cell protrusions. Using
In Vitro systems we demonstrated that Myc-nick directly binds to tubulins and regulates α-tubulin acetylation, by recruiting histone acetyl transferases to microtubules. Because induction of tubulin acetylation, calpain activation and decreased levels of full-length Myc all occur during terminal differentiation, we decided to investigate the role of Myc-nick in cell differentiation. We found that Myc-nick levels are elevated during muscle cell differentiation and in adult mouse muscles such as quadriceps. The ectopic expression of Myc-nick in myoblasts accelerated cell fusion and the expression of myogenic markers. Based on our results, we propose a model for Myc regulation and function during terminal differentiation in which calcium influx promotes calpain activation that in turn converts full-length Myc into Myc-nick. The cleavage of Myc by calpains has 2 roles: first to abrogate the transcriptional blockade to differentiation caused by full-length Myc, and second to generate Myc- nick, which influences cytoskeletal organization and facilitates terminal differentiation.

241/B188
The Expression of Tubulin Cofactor A (TBCA) Is Regulated by a Noncoding Antisense TBCA RNA.
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TBCA is a β-tubulin folding cofactor that is not required for tubulin folding in vitro. However, we showed that TBCA knockdown decreased the amount of α- and β-tubulin, causing G1 cell cycle arrest and cell death in human cell lines. Tbca gene expression was analyzed in different murine tissues being more abundantly expressed in testis, where it is progressively up-regulated from the onset of meiosis reaching the highest levels in differentiating spermatids. The Tbca expression pattern seems to be more associated to microtubule cytoskeleton rearrangements, dynamics and β-tubulin processing through spermatogenesis rather than with meiosis. In this context a close correlation was observed between the Tbca expression waves and the testis specific tubulin isotypes β3 and α3/7, suggesting that the Tbca role in the testis might be associated to β-tubulin isotypes’ maturation and/or interchange. To clarify the Tbca role, we decided to study the expression regulation of the mouse Tbca gene during testis maturation. During these studies we identified two transcribed closely related Tbca genes localized in chromosome 13 and 16 (Tbca13 and Tbca16, respectively). Interestingly, we observed that both genes were expressed constitutively, but they presented opposite patterns during testis maturation. The testis Tbca13 steady state levels increased, whereas those of Tbca16 were progressively decreased from 14 to 25 post-natal days. This data suggested the existence of a regulatory mechanism between the two genes in testis, which prompted us to characterise the respective encoding proteins. Unexpectedly, using mass spectrometry, we were not able to identify the TBCA16 protein in any stage of testis maturation. Thus, we put forward the hypothesis that the Tbca16 transcript was involved in the regulation of Tbca13 gene expression. Indeed we detected that Tbca16 gene is a non-coding anti-Tbca gene, transcribed in a reverse complementary orientation. These data and the fact that the Tbca16 knockdown leads to an increase of the Tbca13 mRNA levels in GC-2spd(ts)-spermatocyte mouse cell line lead us to postulate that Tbca16 is involved in the regulation of the Tbca13 expression during spermatogenesis.

242/B189
Phosphor-Regulation of γ-Tubulin Nucleation Activity in Budding Yeast.
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Phosphorylation has been showed to be an important post-translational modification to modulate the activity of γ-tubulin in microtubule organization. In budding yeast the direct kinase (s) for γ-tubulin (Tub4) has not been proved In Vivo and only one phosphorylation residue (Tyr445) has been confirmed. SerXXX is a putative Cdk1/Cdc28 phosphorylation residue on Tub4p. Site-
directed mutagenesis analysis reveals that SerXXX is an important residue for normal function of γ-tubulin. When SerXXX is mutated to Asp, cells exhibit growth defect with delayed mitosis. SXXXD mutant dies under restrictive temperature instead of arresting. GFP-Tub1 fusion protein is used to monitor the microtubules In Vivo and this demonstrates that SXXXD mutant has longer cytoplasm microtubule. Under restrictive temperature, some microtubules are detached from the spindle pole bodies (SPBs), from which microtubules are nucleated and attached. DAPI staining of the DNA shows that 9.6% of mutant cells have the entire nucleus located into the bud cell. One of the γ-tubulin small complex (gTuSC, the minimal complex for microtubule nucleation) components, Spc97, seems to localize less onto the SPBs. All these data together indicates that phosphorylation on SXXX residue might regulate the nucleation activity of Tub4. Initial analysis indicates that Swe1 and Cdc28 might be involved in Tub4 phospho-regulation as some of the phospho-isofoms are Swe1 or Cdc28 dependent.

243/B190
Interaction of [3H] Halichondrin B with Tubulin.
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Halichondrin B is a sponge-derived, complex natural product that binds in the vinca domain of tubulin and inhibits tubulin assembly. It noncompetitively inhibits the binding of both radiolabeled vinblastine and radiolabeled dolastatin 10 to tubulin. Although halichondrin B has excellent antitumor activity in vivo, scarcity of the natural product led to development of a truncated analog, NSC 707389 (eribulin) that is presently in late clinical trials. To gain greater insight into the interactions of halichondrin B with tubulin, we commissioned preparation of [3H] halichondrin B and have been studying its interactions with tubulin. Scatchard analysis indicated a single class of binding site, but the apparent Ka varied with the protein concentration used. Binding and dissociation reactions were nearly instantaneous, within the limits of the techniques used to study them. We examined inhibitory effects of 16 vinca domain compounds and found that only NSC 707389 and the antimitotic peptides dolastatin 10 and, to a lesser extent, hemiasterlin were strong inhibitors of halichondrin B binding to tubulin. NSC 707389 was a competitive inhibitor (Ki = 0.80 ± 0.3 μM) of halichondrin B binding, while dolastatin 10 was a noncompetitive inhibitor (Ki = 0.35 ± 0.2 μM). In an earlier modeling study, we found that halichondrin B could easily fit into the groove between adjacent αβ-tubulin dimers in the vinca site model, based on the structure of Gigant et al. (Nature 435: 519-522, 2005). This implied that halichondrin B would only bind to aggregates of tubulin larger than the 100 kDa αβ-dimer. However, gel filtration HPLC demonstrated binding of [3H] halichondrin B only to a 100 kDa species. Even when the column was equilibrated with 20 μM halichondrin B, there was no evidence for formation of tubulin species larger than 100 kDa. We have therefore revised our binding model for halichondrin B.

244/B191
Do the β Isotypes of Tubulin Protect Neuronal Cells against Reactive Oxygen Species? J. Guo, R. F. Luduena; Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, TX

Microtubules in neurons are a critical component of the neuronal cytoskeleton. Some evidence suggests that the microtubule network of neurons can be impaired by oxidative stress. is it possible that β tubulin isotypes are involved in this? Reactive Oxygen Species (ROS) are major contributors to oxidative stress. Generation of ROS can be achieved in neuronal cells when NMDA receptors are activated by glutamate/glycine. Undifferentiated SK-N-SH neuroblastoma cells do not express the NMDA receptor, while retinoic acid-induced differentiated SK-N-SH cells do. Here we have shown that ROS were not generated by glutamate/glycine treatment of undifferentiated cells. Levels of β tubulin isotype expression and cell viability were not affected either. However, after glutamate/glycine was applied to differentiated SK-N-SH cells, ROS levels increased greatly, reaching a peak at 30 min, and then decreasing. The expression of both the βII and βIII tubulin isotypes decreased, reaching a minimum at 1 hour, then returning to normal by 24
hr. The expression of the βI isotype increased slightly in the first 2 hours, and then decreased. The mRNA levels of both βII and βIII tubulin increased in the first 2 hours, although they were lower at 30 min than was βI. Interestingly, cell viability was not affected by ROS. We also applied MK801, an NMDA receptor inhibitor, to the differentiated cells before glutamate/glycine treatment and found that ROS levels change only slightly. β isotype expression and cell viability were not affected either. These results suggest that β tubulin isotype levels can be affected directly by excessive ROS generation in the cells. Cells are viable regardless of ROS generation. However, when glutamate/glycine was applied to differentiated cells in which the β isotype levels had been lowered by siRNA knock-down, very different results were obtained. The viability of cells treated with siRNAs for βII and βIII, decreased greatly in 1 hour, later recovering. The effect was particularly large when βIII was knocked down. Our results suggest that βII and βIII, but especially βIII, play an important role in protecting neuronal cells from oxidative stress.

245/B192
TBCE and TBCB Share Molecular Recognition Mechanisms with +Tips.
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Tubulin folding cofactors B and E are two CAP-gly containing proteins that participate in the folding and dimerization pathways, as well as the dissociation process of the tubulin heterodimer. This way, the TBCE-TBCB complex could behave as a quality control mechanism for alpha-tubulin monomers, being capable of selecting individual alpha-tubulin subunits for recycling or degradation. The presence of a UBL domain in both cofactors supports the fact that these proteins could participate in the removal from the cell of unsuitable tubulin monomers. TBCE and TBCB do also contain a CAP-Gly domain despite they have never been shown to localize on the tips of microtubules as other CAP-Gly containing proteins. We have investigated by means of biophysical (circular dicroism spectroscopy, dynamic light scattering and analytical centrifugation) and biochemical (native electrophoresis of purified proteins and the complexes formed) methods the molecular mechanism implicated in the association of TBCB with TBCE into the binary complex that efficiently recognizes and dissociates the tubulin heterodimer. We also show how that both, TBCE and TBCB localize on the vicinities of the mitotic spindle and that TBCB does indeed localize on the plus ends of mitotic microtubules.

246/B193
Identification of New Tubulin Acetylation Modifying Enzymes by High-Throughput siRNA Screening.
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One of the several types of post-translational protein modifications affecting the tubulins is the acetylation of alpha-tubulin at Lys40. The contribution of tubulin acetylation on dynamic instability of the microtubules, microtubule-dependent cellular processes, and anti-microtubule drug response are unclear. Similarly, the identity of enzyme(s) responsible for tubulin acetylation remains to be determined while a few tubulin deacetylating enzymes such as HDAC6 and Sirt2 have been described. In search for novel modulators of tubulin acetylation we performed a high-throughput siRNA screen utilising the Cell Spot Microarray (CSMA) technology. CSMA is based on a reverse transfection method, where individual gene silencing siRNAs are printed onto a microarray plate in a spot format. Tissue culture cells of interest are cultured on top of the individual siRNA spots and due to the specific matrix, they only adhere and grow onto the siRNA spots. Each cell island consists of 50-200 cells and up to 46000 individual gene silencing assays can be performed simultaneously allowing genome-wide analysis in a single assay plate. In our assay, the endpoint measurement was antibody-based detection of tubulin Lys40 acetylation. The
primary screen carried out using an epigenetic siRNA library consisting of 1328 siRNAs against known epigenetically active protein domains led to the identification of novel tubulin modifying genes. Validation of the hit genes and their functional characterisation is currently ongoing as well as extension of the study towards genome-wide siRNA screen. We expect the results of this study to provide new insights into the roles of tubulin acetylation in cancer biology and tumour chemosensitivity.

247/B194
Structural Mechanisms Benzimidazole Interactions with β-Tubulin.
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Benzimidazoles are widely used as fungicides, herbicides and antihelminthic drugs due to their high affinity to tubulins of different origin. However, precise localization of benzimidazole binding site on tubulin molecular surface is still not described in details. For accurate prediction of benzimidazole binding site we used combination of several approaches: analysis of spatial distribution of tubulin mutations conferring benzimidazole resistance, analysis of benzimidazole structural peculiarities that are responsible for site recognition, reconstruction of spatial structure of fungal (Neurospora crassa and Mycosphaerella graminicola) and animal (Haemonchus contortus and Homo sapiens) β-tubulin complexes with albendazole (antihelminthic drug) and carbendazim (antifungal and antihelminthic drug) and further estimation of their stability via long-time (30 ns) molecular dynamics calculation. Accordingly to obtained data, β-tubulin benzimidazole binding site contains amino acids in positions 152, 156, 163-165, 167, 195-198, 200, 236, 250, 251 and 253. Ligands are buried into molecule enough deeply, what is typical for benzimidazole derivatives with affinity for other proteins. Conformation energy of studied ligands in binding sites are stable less than in free state. Energy downshift is within range from 48.6 kJ/mol for β-tubulin complex from M. graminicola with albendazole to 88.5 kJ/mol for tubulin complex from N. crassa with carbendazim. Average level of molecular oscillation for albendazole decreases from 2.5 Å to 0.7 Å. This value practically doesn’t change for carbendazim, but amplitudes of oscillation for both ligands diminish approximately 10 fold. Thus, described site can be considered as the most favorable for interaction between β-tubulin and benzimidazole molecules. This work is supported by cooperative STCU-NASU grant 4929 "Application of grid-resource for total analysis of β-tubulin spatial structure features causing a different sensitivity to benzimidazoles".

248/B195
Beta Tubulin Class II Protein Increases with Low Dose Paclitaxel Treatment in MDA-MB-231 Breast Cancer Cells.
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Taxanes are commonly used in combination chemotherapy for solid tumors. These drugs bind to beta tubulin, stabilize microtubules in mitotic spindles and arrest cells in G2/M phase of the cell cycle. There are 8 genes that code for beta tubulins and the gene products are classified into seven isotype classes based upon their carboxyl terminal sequences. Unfortunately, the effectiveness of taxanes is reduced because some tumors are initially resistant or become resistant to taxanes after repeated cycles of chemotherapy. It is possible that changes in amounts of beta tubulin isotypes or microtubule interacting proteins (MIPs) could contribute to drug resistance. We investigated changes in tubulin isotypes and MIPs in response to paclitaxel in MDA-MB-231 breast cancer cells. We demonstrated previously that this cell line expresses primarily beta tubulin classes I, IV and V (Hiser et al., 2006, Cell Motil Cytoskel, 63:41-52). Using Western blotting and isotype-specific antibodies, we found that beta tubulin class II protein increases 2-3 fold after 16 hours treatment with 4 nM paclitaxel. Comparative real time PCR did not reveal a comparable change in mRNA for beta tubulin class II genes, TUBB2A or TUBB2B. Flow cytometry demonstrated that this increase in beta tubulin class II occurs prior to cell cycle...
synchronization and G2/M arrest. This result suggests that paclitaxel treatment leads to changes in either beta tubulin class II mRNA or protein stability. An increase in stathmin protein was also found over the same time period. Western blotting and comparative real time PCR did not show similar increases in tubulin isoform levels for tubulin isoform classes I, III, IV or V or MAP4.

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249/B196

How Does Nucleotide Content Regulate Microtubule Mechanics?
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Various studies over the last decades have found that the stiffness of microtubules is a function of their length (Kurachi et al., 1995, Cell Motil. Cytoskeleton 30:221-228; Kis et al., 2002, PRL 89: 248101; Pampaloni et al., 2006, PNAS 103:10248 -10253). This length dependence has been successfully reproduced by mechanical models that take into account the molecular architecture of microtubules and allow for sliding of protofilaments relative to one another during bending (Heussinger et al., 2007, PRL 99:048101). In these models, the characteristics of the length dependence are determined by the bending stiffness of the protofilaments and the strength of protofilament interactions. Consequently, measurements of changes in microtubule mechanical properties, e.g. caused by microtubule binding proteins or other agents, can yield information on how the agent affects the molecular interactions of the tubulin dimers in the microtubule lattice. Based on high precision thermal fluctuation measurements on fluorescent tracer beads attached to grafted microtubules, we present results for the stiffness, relaxation times and friction parameters for microtubules polymerized from the slowly-hydrolyzable GTP analogue GMPCPP. In contrast to taxol-stabilized microtubules which show a pronounced length dependence of the stiffness that is attributed to weak protofilament interactions (Taute et al., 2008, PRL 100:028102), the stiffness of GMPCPP microtubules is constant in the measured length range of 4-25 µm. In agreement with structural data (Chang and Nogales, 2005, Nature 435:911-915), our results hence indicate that the coupling between protofilaments is much stronger in GMPCPP microtubules than in taxol-stabilized GDP microtubules, thereby supporting the notion that microtubule flexibility can be regulated by the nucleotide content of the polymer. Surprisingly however, the thermal fluctuation dynamics in the short length regime show evidence for internal friction contributions similar to those found for taxol microtubules.

250/B197

Regulation of Tubulin-Membrane Interaction by Tubulin’s Carboxy-Terminal Tails.
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Microtubules and their subunit protein, the tubulin heterodimer, are components of the aqueous compartment of cells, and indeed aqueous solutions of tubulin at 50 mg/ml or greater are readily obtained. This is not surprising since tubulin is a highly negatively charged protein, largely due to the 10-15 residue long, glutamic acid-rich, unstructured carboxy-terminal tails (CTT) on both subunits. Nonetheless, tubulin has been reported as a component of purified membrane preparations for many years. Association of tubulin with membranes has included plasma membranes, intracellular membranes such as Golgi and mitochondrial outer membranes, and purified lipid membranes. The exact nature of the tubulin-membrane interaction has not been defined, nor has it been shown that all such interactions rely on the same underlying mechanism. In several cases, tubulin docking with lipid-embedded membrane proteins has been invoked. One such case is tubulin binding to the mitochondrial outer membrane. We have shown that tubulin binds to VDAC in the outer membrane through the CTT, with functional consequences for mitochondrial function. Thus tubulin’s CTT can promote membrane interaction by promoting binding to embedded membrane proteins. Tubulin has also been shown to bind to lipid membranes devoid of other proteins. We show by charge-shift electrophoresis and non-ionic detergent extraction that the presence of the CTT inhibits direct interaction of tubulin with amphiphiles. Thus, tubulin CTT can enhance or inhibit interaction with membranes, but these effects are mediated through two different molecular mechanisms.
**Microtubule-Associated Proteins I (251 – 265)**

**251/B198**

**Growth-Arrest-Specific Protein 2 Arrests Cell Division by Disrupting Microtubule Dynamics.**

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The growth-arrest-specific 2 (gas2) gene was initially identified from high levels of expression in murine fibroblasts under growth arrest conditions. It was also found that gas2 is down-regulated upon serum stimulation, which leads to re-entry into the cell cycle. It has been shown that the gas2 gene product-Gas2 protein, may interact with both F-actin and microtubules. In this study, full-length Gas2 protein was microinjected into *Xenopus laevis* embryos, and found to prevent subsequent cell divisions. Cryo-confocal microscopy analysis also suggested that Gas2 overlaps with microtubules. Electron microscopy further revealed that the purified full-length Gas2 can bundle microtubules. Furthermore, a cytokinesis analog contractile array assay showed that Gas2 co-localizes with double F-actin rings during the cell wound healing, which is a typical phenotype seen when the *Xenopus laevis* oocytes are treated with microtubule stabilization drug Taxol. Structure function analysis demonstrated that Gas2 can stabilize microtubules via its c-terminal Gas2 domain. Flow cytometry has shown that Gas2 DNA transfected HeLa cells have a significant increase in multiple nuclei cells in the culture, which suggests that over-expression of Gas2 can result in cytokinesis failure, and the cell cytokinesis failure is caused by the effect of Gas2 on microtubule dynamics.

**252/B199**

**MAP1B Regulates Axon Formation by Promoting TIAM1 Association with Microtubules and Modulating RAC1/Cdc42/Rhoa Activities.**

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Cultured neurons obtained from MAP1B-deficient mice have a delay in axon outgrowth and a reduced rate of elongation. We now show that MAP1B deficiency causes a significant decrease in Rac1 and cdc42 activity, along with an increase in Rho activity. MAP1B deficiency also prevents the binding of Tiam1, a guanosine nucleotide exchange (GEF) factor for Rac1, to microtubules. The decrease in Rac1/cdc42 activity is paralleled by decreased LIMK activity and reduced cofilin phosphorylation. The expression of a constitutive active form of Rac1 or of Tiam1 -a Rac1 GEF- rescue the axon growth defect of MAP1B-deficient neurons. Taken together, these observations, define a new and crucial function for MAP1B during neuronal polarization, which is required for an efficient crosstalk between microtubules and the actin cytoskeleton.

**253/B200**

**MT Severing Activity Scales Spindle Length in Xenopus Egg Extract.**

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The *Xenopus laevis* metaphase spindle is a complex and dynamic steady state microtubule (MT) structure for which the physiological determinants of size and shape are poorly understood. To elucidate mechanisms of mitotic spindle assembly and scaling, we used a Brownian Dynamics numerical simulation to model a two-dimensional bipolar MT structure using a minimal set of protein components. Incorporating experimentally measured parameters, we found that focusing
of MT minus ends at spindle poles depended not only on motor-driven movements and minus end cross-linking, but also required depolymerization at the poles, and that altering this parameter changed spindle length. To test whether this mechanism contributes to the differential scaling of larger mitotic spindles in X. laevis compared to the smaller spindles in X. tropicalis egg extracts, we evaluated the stability of fluorescently-labeled, Taxol-stabilized MTs in extracts using a quantitative flow cell assay. We found that Katanin-dependent MT severing, but not Kinesin-13 dependent MT depolymerization, was significantly greater in X. tropicalis extracts. Although Katanin levels and pole localization were similar, extract-mixing experiments revealed that, like spindle length, severing activity was titratable and dose dependent in the two extracts. Katanin inhibition by antibody addition increased spindle length in both extracts and had a much more dramatic effect on the smaller X. tropicalis spindles, as kinetochore fibers (k-fibers) lengthened, causing centrosomes to fall off and disrupting pole structure. Introducing hyperstable k-fibers to numerical simulations reproduced this phenotype and predicted that spindle poles could withstand only small numbers of hyperstable k-fibers, which was confirmed by Katanin inhibition in spindles with reduced numbers of chromosomes. Thus, a combination of modeling and experimental approaches reveals mechanisms of spindle morphogenesis in Xenopus egg extracts.

254/B201
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We recently used a MT co-sedimentation assay, combined with 1D and 2D PAGE and mass spectrometry, to identify 270 microtubule associated proteins (MAPs) from early Drosophila embryos. A bioinformatics analysis of this data set, based on binary protein interaction data, yielded a putative interaction network of MAPs. We have extended this data set to include Drosophila homologues of MAPs identified in other organisms, including plants, mouse and humans. The addition of homologues and interologs of MAPs leads to a relatively highly clustered network, 92% of which are fully connected. Network statistics indicate that the additional proteins added to the fly set are functionally relevant. This bioinformatics analysis identifies a complex network of putative interactions, which self organises into clades corresponding to known protein function, linked by key proteins. We have begun to assess the functional validity of our approach using sensitised RNA-interference in Drosophila S2 cells, in which centrosome-driven microtubule nucleation is knocked-down. A preliminary analysis of key candidate proteins suggests that at least some of these previously uncharacterised MAPs do, indeed, possess roles in MT organisation during mitosis. Together, our results reinforce the suggestion that a bioinformatics approach based on yeast two-hybrid data represents functionally relevant interactions, and identifies new MAPs whose roles in S2 cells are normally masked by the dominant role of centrosomes in microtubule nucleation.

255/B202
Phosphoregulation of the Budding Yeast EB1 Homolog Bim1p by Aurora/Ipl1p.
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EB1-like proteins are conserved regulators of microtubule dynamics in all eukaryotes. They target growing microtubule plus ends in Vivo and deliver associated factors to regulate the interactions of microtubules with cellular structures such as kinetochores or cortical attachment sites. Despite their widespread importance, molecular mechanisms regulating the activity of these proteins have largely remained elusive. Here we show that the budding yeast EB1 protein Bim1p is regulated by AuroraB/Ipl1p phosphorylation of its linker domain. Biochemical analysis has revealed that Bim1p directly associates with the Ipl1p kinase and, upon activation by Sli15p, becomes phosphorylated on a cluster of six sites in the flexible linker domain. Bim1 phosphorylation occurs during anaphase in vivo, and it is required for normal spindle elongation kinetics and an efficient
disassembly of the spindle midzone. Recombinant Bim1p phosphorylated with Ip1/Sli15 In Vitro or a mutant mimicking constitutive phosphorylation display a reduced affinity for taxol-stabilized microtubules. We have reconstituted the interaction between Bim1p and dynamic microtubules using total internal reflection fluorescence microscopy (TIRF) in vitro. Bim1p autonomously tracks growing microtubule plus ends and this activity requires the dimeric molecule. Our analysis indicates that the Bim1p linker domain is critical for MT binding, autonomous plus end tracking, and suggest a mechanism for the use and regulation of CH domains in an EB1 protein. Moreover, we are currently creating Ip1 mutants specifically affected in the interaction with Bim1p. These mutants allow us to investigate the role of Ip1 plus end targeting in vivo.

256/B203
The Microtubule-Associated Rho Guanine Nucleotide Exchange Factor GEF-H1 Interacts with Exocyst and Regulates Vesicle Trafficking.
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The Rho-related small GTPases are involved in regulation of cell polarity and motility through their effects on the cytoskeleton, cell adhesion and membrane trafficking. They have been implicated in the regulation of both endocytic and exocytic membrane traffic by virtue of their capability to organize coordination between actin and microtubule cytoskeletons. Herein, we present evidence that GEF-H1, a Rho-specific nucleotide exchange factor that is regulated by its interaction with microtubules (MT) is involved in regulating vesicle trafficking. Depletion of GEF-H1 using siRNA leads to accumulation of large vesicles in the perinuclear region in Hela cells indicative of disruption in vesicular transport. GEF-H1 regulates membrane traffic by modulating Rho activity, since mutant deficient in catalyzing the nucleotide exchange was not able to rescue the knockdown phenotype. Expression of a dominant negative RhoA construct induces a similar block of vesicle transport. We determined that at least a sub-set of the accumulated vesicles in GEF-H1-depleted cells are recycling endosomes as they stained with Rab11, a small GTPase associated with the pericentriolar recycling compartment. Furthermore, GEF-H1-depleted cells show enhanced accumulation of labelled transferrin (Tfn) in punctate structures throughout the cytoplasm and at the cell periphery. We also observed a delay in recycling of labelled Tfn to the surface, indicating that GEF-H1 likely regulates the recycling of the transferrin receptors. Of particular interest, we find that GEF-H1 physically interacts with Sec5, Exo84 and Exo70, components of an evolutionarily conserved complex called the exocyst, known to be involved in tethering vesicles to the plasma membrane, further implicating GEF-H1 in vesicle transport. Furthermore localization of Exo70 and Sec6 to the plasma membrane is affected by GEF-H1 depletion indicating that latter might regulate vesicle trafficking by mediating the localization and/or function of the exocyst complex. Together these studies indicate that the Rho exchange factor GEF-H1 might be an ideal candidate for coordinating vesicular transport with regulation of the microtubule and/or actin cytoskeletons.

257/B204
Two Members of the ASE1/PRC/MAP65 Family Are Involved in MT Bundling and Cortical Array Organization in Arabidopsis.
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Microtubules are organized into complex arrays that are required for critical cellular processes including cell division and morphogenesis. Bundled microtubules are important components of arrays. Ase1/PRC/MAP65 are microtubule bundling proteins predicted to play important roles in array organization. We investigated two of the nine MAP65 gene family members in Arabidopsis thaliana to define their specific roles in interphase microtubule bundling. In the plant cortical array, microtubule bundles form when the plus end of a treadmilling microtubule encounters another polymer and then changes growth trajectory to align parallel with the other microtubule. The probability of bundling is dependant upon the angle in which the two polymers interact. Live-cell observations of MAP65-1 and -2 fluorescent protein fusions indicate localization only to regions of
anti-parallel bundling within the cortical array. Double mutants (map65-1/map65-2) show growth defects relative to wildtype and single mutant plants suggesting that cortical array organization is impaired in these plants. Observations of microtubule behavior in the map65-1/map65-2 mutants suggest that MAP65-1 and -2 are important for both the initial bundling interaction and the maintenance of the bundle once formed.

258/B205
Heterodimerization of Mammalian End Binding Proteins.
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Microtubule plus-end tracking proteins (+TIPs) are involved in many microtubule-based processes including regulation of chromosome segregation and maintenance of cell polarity. End binding (EB) proteins are highly conserved +TIPs that play a pivotal role in regulating microtubule plus ends and in organizing dynamic +TIP networks. Here we have analyzed in detail the dimerization properties of the three mammalian EB proteins, EB1, EB2 and EB3, using a combination of methods. Based on Förster resonance energy transfer we demonstrate that the C-terminal dimerization domains of EBs (EBc) which are targeted by numerous +TIP binding partners readily exchange their chains in solution and document that EB1c and EB3c preferentially form heterodimers. In contrast, EB2c does not participate significantly in any heterotypic complexes. Thermodynamic and kinetic experiments in combination with modeling and mutagenesis reveal that a structural property controls homo- versus heterodimer formation of EBc domains. Fluorescence and nuclear magnetic resonance studies in the presence of the CAP-Gly domains of either CLIP-170 or p150glued, or a fragment derived from APC show that chain exchange of EBs can be differentially controlled by +TIP binding partners. We extend our observations on the EBc domains to full length EB1 and EB3 and report that both proteins exchange their chains in solution and are capable of forming heterotypic complexes. Live cell imaging provides evidence that heterodimer formation between EB1 and EB3 but not between EB2 and EB3 is a mechanism occurring in cells. Together, our findings provide a molecular basis for understanding the dominant negative effect of C-terminal EB domains and expand our knowledge on the structural and functional properties of the remarkable family of EB proteins.

259/B206
Probing Interactions between CLIP-170, EB1, and Microtubules.
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CLIP-170 is a microtubule (MT) plus-end tracking protein (+TIP) that dynamically localizes to the MT plus end and regulates MT dynamics. The mechanisms of these activities remain unclear because the CLIP-170-MT interaction is poorly understood, and even less is known about how CLIP-170 and other +TIPs act together as a network. CLIP-170 binds to the acidic C-terminal tail of α-tubulin. However, the observation that CLIP-170 has two-CAP-Gly motifs and multiple serine-rich regions suggests that a single CLIP-170 molecule has multiple tubulin binding sites, and that these sites might bind to multiple parts of the tubulin dimer. Using a combination of chemical cross-linking and mass spectrometry, we find that CLIP-170 binds to both α- and β-tubulin, and that binding is not limited to the acidic C-terminal tails. We provide evidence that these additional binding sites include the H12 helices of both α- and β-tubulin and are significant for CLIP-170 activity. Previous work has shown that CLIP-170 binds to the +TIP EB1 via the EB1 C-terminus, which mimics the acidic C-terminal tail of tubulin. We find that CLIP-170 can utilize its
multiple tubulin binding sites to bind to EB1 and the MT simultaneously. These observations help
to explain how CLIP-170 can nucleate MTs and alter MT dynamics, and they contribute to
understanding the significance and properties of the +TIP network.

260/B207
Death-Associated Protein Kinase Activates MARK2/Par-1 to Regulate Microtubule
Dynamics and Tau Toxicity.

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Death-associated protein kinase (DAPK) is a death-promoting, Ser/Thr kinase and is abundantly
expressed in neurons. DAPK has been linked to several modes of neuronal injury and implicated
in late-onset Alzheimer’s Disease (AD). In addition to its well-documented function in regulating
actin cytoskeletons, we report here that this kinase elicits an inhibitory role in microtubule (MT)
assembly. This effect of DAPK is mainly mediated by its activation of the microtubule affinity-
regulating kinase 2 (MARK2), which is known to phosphorylate tau and related microtubule-
associated proteins and thereby to cause MT destabilization. Intriguingly, the death domain of
DAPK, but not its catalytic activity, is both sufficient and required for MARK2 activation by
interacting with MARK2 spacer region, thereby relieving an autoinhibition mechanism of MARK2.
The DAPK-enhanced MARK2 activity to promote Tau phosphorylation at S262 (MARK2
consensus site) is observed in hippocampal neurons and, moreover, DAPK knockout mice have
reduced level of tau phosphorylation at this site. Using a Drosophila model for studying tau
toxicity, we show that DAPK acting through the Drosophila MARK2 homology Par-1 to trigger a
tau-mediated rough-eye phenotype. Furthermore, DAPK enhances tau toxicity through a S262-
dependent manner. Our findings reveal not only a new function of DAPK in regulating MT
dynamics but also a molecular link of DAPK to tau phosphorylation, an event associated with AD
pathology.

261/B208
The Doublecortin Family of Maps Stabilise Both Lateral and Longitudinal Contacts
between Tubulin Dimers.

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During brain development, immature neurons grow neurites and migrate to form the highly
ordered layers of the cortex. The doublecortin (DCX) family of microtubule-associated proteins
(MAPs) was found to be essential for this process. Single point mutations in DCX were found to
cause severe neuronal migration disorders in humans. Previous studies have opened the way to
an understanding of the relationship between the structure of DCX and its impact on microtubule
dynamics, the details of which are still being explored. DCX is a 40 kDa protein with a tandem of
11 kDa globular microtubule binding domains (called DC domains) and a C-terminal
Serine/Proline rich domain. Doublecortin-Like Kinase (DCLK) is a close homologue and has a 40
kDa N-terminal domain with 70% identity with DCX and a C-terminal 40 kDa kinase domain; its
function, however, remains unclear. Here we report biochemical evidence that DCL (DCX-Like,
splicing variant of DCLK lacking the kinase domain) nucleates and binds microtubules like DCX.
A previous study based on cryo-electron microscopy (cryoEM) and helical reconstruction showed
how a truncated construct of DCX stabilised the microtubule lattice by bridging adjacent
protofilaments with a DC domain, a unique mechanism among neuronal MAPs. Using cryoEM and
single particle algorithms we have generated reconstructions of 13-protofilament
microtubules co-polymerised in presence of full-length DCX. We show that a DC domain binds at
the interface between 4 tubulin heterodimers, in a way that apparently does not interfere with
kinesin binding, in good agreement with previous reports. Complementary biochemical
characterisation revealed tubulin C-terminal tails are not required for the DCX-tubulin interaction. These results provide insight into the complex interplay between DCX-like MAPs, tubulin and kinesin in neurons.

262/B209
Interactions between EB1 and Microtubules: Dramatic Effect of Affinity Tags and Evidence for Cooperative Behavior.
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Plus end tracking proteins (+TIPs) are a unique group of microtubule binding proteins that dynamically track microtubule (MT) plus ends. EB1 is a highly conserved +TIP with a fundamental role in MT dynamics, but it remains poorly understood in part because reported EB1 activities have differed considerably. One reason for this inconsistency could be the variable presence of affinity tags used for EB1 purification. To address this question and establish the activity of native EB1, we have measured the MT binding and tubulin polymerization activities of untagged EB1 and EB1 fragments and compared them to those of his-tagged EB1 proteins. We found that N-terminal his-tags directly influence the interaction between EB1 and MTs, significantly increasing both affinity and activity, and that small amounts of his-tagged proteins act synergistically with larger amounts of untagged proteins. Moreover, the binding ratio between EB1 and tubulin can exceed 1:1, and EB1-MT binding curves do not fit simple binding models. These observations demonstrate that EB1 binding is not limited to the MT seam, and they suggest that EB1 binds cooperatively to MTs. Finally, we found that removal of tubulin C-terminal tails significantly reduces EB1 binding, indicating that EB1-tubulin interactions are mediated in part by the same tubulin acidic tails utilized by other MAPs. These binding relationships are important for helping to elucidate the complex of proteins at the MT tip.

263/B210
DTBCB: A New Drosophila Map Controlling MT Dynamics and Cell Polarity during Development.
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The microtubule (MT) network is a core component of the cell cytoskeleton controlling fundamental processes as chromosomes segregation or polarized transport. These biological polymers are polarised with a stable, often anchored, minus end and a dynamic plus end. In the drosophila oocyte, the MT network is crucial to localise the polarity determinants that will generate both the antero-posterior and dorso-ventral axis of the adult fly. In order to identify genes required for the proper organisation of MTs in the oocyte, a genetic screen for polarity defects was previously carried out in the lab. We mapped one of the mutations identified to the uncharacterised CG11242 gene, which is the ortholog of the Tubulin Binding Cofactor B (TBCB) mammalian gene. Mammalian TBCB was reported In Vitro as being part of a chaperone complex required for αβ Tubulin heterodimer formation. We showed in S2 cells and fly embryos, that dTBCB is localised to the centrosome and along the MT network. We confirmed In Vitro by MT pull down that dTBCB could directly interact with MTs, showing that dTBCB is a microtubule-associated protein (MAP). We therefore analysed the microtubule network morphology in dTBCB mutant fly tissues. In the oocyte as well as in the surrounding epithelial cells, the mutation caused a very strong MT destabilisation, which correlated with severe polarity defects. In order to study the precise role of dTBCB association with the MTs independently of its α-Tubulin folding role, we incubated recombinant dTBCB with purified folded αβ Tubulin and measured the dynamic parameters of individual MTs. In this In Vitro assay, dTBCB enhances MT polymerisation speed as well as catastrophe rate, thereby making the MTs more dynamic. Hence dTBCB appears to function like some MT plus end tracking proteins such as EB1, to which we observed a direct binding with dTBCB. In summary, we propose that CG11242 codes for an ortholog of the
mammalian chaperone TBCB and identify a new function for this gene: dTBCB regulates MT dynamics In Vivo and In Vitro through a direct association with MTs, thereby controlling cell polarity during drosophila development.

264/B211
Differences in Microtubule Attachment at the Yeast Spindle Pole Body.
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Microtubules (MTs), dynamic structures involved in chromosome separation and spindle orientation, are anchored at the spindle pole body (SPB), the MT organizing center in yeast. The cytoplasmic MTs (cMTs) of budding yeast are responsible for the proper positioning and orientation of the mitotic spindle. A synthetic genetic array performed with a temperature-sensitive allele of Stu2, a MT associated protein thought to be involved with MT anchorage at the SPB, identified SHE1, a relatively uncharacterized gene. When SHE1 is deleted, cMTs detach from the SPB and track the periphery of the cell before depolymerizing. The detachment of cMTs in she1Δ mutants could stem from either a weakened connection at the SPB or an increase in pulling forces from MT motors. She1 has been shown to regulate the timing of the loading of dynactin components onto cMTs, which are required for dynein function. The motion of detached cMTs around the cortex of the cell is thought to be dynein dependent, as deletion of NIP100, a dynactin complex protein, rescues the detachment phenotype. SPB mutants that also detach cMTs have increased rates of cMT release when coupled with she1Δ, suggesting separate mechanisms. Additionally, attempts at finding any interactions with She1 at the SPB have all been negative. However, additional pulling forces cannot fully explain the detaching of cMTs because careful examination of the detachment phenotype in she1Δ asynchronous culture reveals that cMTs do not detach during anaphase when dynein is normally active. Arresting she1Δ cells in the mating phase increases the frequency of detachment. at this stage, as well as during G1, cMTs are anchored at the half-bridge of the SPB, as opposed to the outer plaque during mitosis. This has led us to the hypothesis that the MT anchoring complex at the half-bridge is weaker than that of the outer plaque and that part of She1’s function is to curb the pulling force of dynein during this part of the cell cycle. Cytoplasmic MTs can then remain attached and properly orient and position the nucleus for division.

265/B212
Regulation of Bik1p.
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Bik1p is the yeast homologue of the mammalian CLIP170 protein and is a member of the CAP-Gly protein family of microtubule binding proteins. Bik1p stabilizes microtubules and in its absence, microtubules are very short. In yeast two major pathways, the Kar9p and dynein pathways, determine accurate spindle positioning. These pathways carry out distinct functions for spindle positioning, with the Kar9p pathway orienting cytoplasmic microtubules and the dynein pathway pulling the spindle across the bud neck. In addition to playing a key role in the dynein pathway, Bik1p also interacts with Kar9p. However, the function of its interactions with the Kar9p pathway and how these interactions regulate the function of Bik1p are not completely understood. To better understand the mechanisms of interaction between the two spindle positioning pathways, a screen was carried out to identify bik1 mutants lacking the Kar9p-Bik1p interaction. This resulted in the identification of the Y61H mutant, which contains a tyrosine to histidine mutation at residue 61. This mutation results in weaker Kar9p-Bik1p and Kip2p-Bik1p interactions. Y61H displays short microtubules, and the fluorescent-tagged protein displays an altered localization. The protein encoded by Y61H is about 8 kD smaller than Bik1p indicating that this mutation affects the stability of the protein or prevents a post-translational modification.
Cilia and Flagella I (266 – 289)

266/B213

LC8-Mediated Assembly Mechanism of Flagellar Complexes.

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The assembly of the axoneme inside cilia and flagella is an extraordinary undertaking. The components are synthesized in the cell body whereas assembly occurs at the tip of these slender organelles. In general, the components are first packaged into precursor complexes and then delivered to the flagellar tip to be converted into mature complexes, ultimately becoming part of the precise, elaborate axonemal nanomachine; however, the details of this process are poorly understood. To study this problem of assembly, we focus on two homodimeric molecules in Chlamydomonas radial spoke (RS) complex: RSP3 and LC8 that modulate various target proteins to promote assembly of various molecular complexes. RSs are absent in the axonemes of the RSP3 and LC8 mutants, yet curiously soluble precursor and mature RSs are present inside the flagella of LC8 mutants, suggesting a deficiency in RS docking. Interestingly, adjacent to the N-terminal axoneme-binding site of RSP3 are three conserved LC8-binding motifs aligned in tandem. We postulate that RSP3 is an LC8 target protein and their interaction is critical for RS assembly. To test this, wild type axonemes were treated with trypsin, digesting RSs into fragments. As revealed by 2-D native gel/SDS-PAGE and western blots, LC8 co-migrates with RSP3 N-terminal fragments when most of the other RSPs are not detectable. Likewise, the N-terminal fragment expressed in Chlamydomonas is assembled along with LC8 into axonemes even though the majority of RS proteins are absent. Importantly, while LC8 is present in mature RSs, it is absent from the precursor complex. These results suggest that LC8 dimers and RS precursors are delivered separately to the flagella and that the tip an array of LC8 binds to the RSP3 dimer to transform the precursor complex into a rigid stalk that docks to the outer doublets.

267/B214

Role of EB1 and EB3 in Ciliary Assembly in Human Cells.

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We previously showed that the MT plus-end tracking protein EB1 is required for assembly of primary cilia in mouse fibroblasts (Schroeder et al. Curr. Biol. 2007), but the mechanism by which EB1 affects ciliogenesis remains unknown. In mammalian cells two additional members of the EB protein family (EB2 and EB3) are present, but their role in ciliogenesis has not previously been investigated. In order to gain further knowledge about the role of EB proteins in cilia formation we depleted EB1, EB2, or EB3 from Human Foreskin Fibroblast (hFF) and retinal pigment epithelial (RPE) cells using siRNA, and analyzed the effect of this depletion on primary cilia formation in growth-arrested cells using immunofluorescence microscopy (IFM). Consistent with our previous results we found that depletion of EB1 inhibits ciliogenesis by ca. 50% in growth-arrested cells. Furthermore, we found that depletion of EB3, but not EB2, had a similar inhibitory effect on cilia formation. These results were confirmed by IFM analysis of RPE cell lines stably expressing GFP-tagged full-length or truncated (dominant negative) EB1, 2, or 3. Preliminary transmission electron microscopy (TEM) analysis of serum-starved mock-transfected or EB1-depleted hFF cells showed that vesicles appear to accumulate in the vicinity of the basal body when EB1 is depleted. However, IFM analysis of EB1-, 2-, or 3-depleted cells using antibodies specific for the Golgi protein GMAP210 revealed no significant differences in the overall localization or organization of the Golgi as compared to mock-transfected cells. These results suggest that the defect in ciliogenesis resulting from depletion of EB1 or EB3 is not due to a general defect in the Golgi, but may result from a more specific defect in the trafficking of vesicles to the ciliary base.
We are currently addressing this issue further using TEM analysis as well as GST pull-down assays to identify binding partners of EB1 in the cilium Basal body.

268/B215
Origin of the Cilium: Novel Approaches to Examine a Centriolar Evolution Hypothesis.
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Recently, a new hypothesis was proposed regarding the evolution of the cilium from an enveloped RNA virus (Satir et al., 2007 Cell Motil. Cytoskeleton 64:906). The hypothesis predicts that there may be specific centriolar or basal body RNAs with sequences reminiscent of retroviruses, and/or that the nuclear genes for certain centriole-specific proteins would have viral origins. Four independent laboratories have reported the existence of centrosomal RNA (cnRNA). Methods for studying cnRNA are described. We analyzed evidence of relatedness of known full length cnRNAs to extant viral molecules. Out of 14 cn RNAs studied, 12 have similarity to entries in viral databases, all but one with E-values of ≤1e-4. Some centrosomal, and possibly uniquely centriolar, proteins also have protein relatives in viral databases that meet criteria accepted to indicate a relationship by descent. Nine general cytoskeleton proteins exhibited no significant similarity to viral proteins. The speculation that centrioles are invaders of RNA viral origin in the evolving eukaryotic cell is strengthened by these findings.

269/B216
Remarkable Conservation of Non-Canonical Axoneme Structures from Parasites to Mammals.
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Cilia and flagella are generally classified into ‘9+2’ motile and ‘9+0’ immotile sensory cilia, but the true diversity of ciliary structures and functions across eukaryotes is becoming increasingly apparent. Promastigote forms of the Leishmania parasite utilize their 9+2 flagellum during their passage through the insect vector for locomotion, cell morphogenesis, attachment to insect vector epithelial cells and, most likely, sensory functions. The amastigote forms that infect macrophages of the mammalian host are often wrongly described as being ‘aflagellate’ despite the presence of a small flagellum that protrudes a short distance from the flagellar pocket. Why do amastigotes retain a short flagellum at all? Using electron microscopy and 3D electron tomography to analyse the structure of amastigote flagella at high resolution, we have uncovered a striking similarity between the microtubule axoneme structure of the Leishmania parasite infecting a macrophage and vertebrate sensory cilia. Both have nine microtubule doublets but in an unusual ‘collapsed’ symmetry. 3D reconstructions reveal that in both cases it is not a particular doublet that breaks the symmetry and moreover the initial collapse often involves two doublets. The tip of the Leishmania flagellum was frequently in intimate contact with the macrophage vacuole membrane, suggestive of a ‘parasitophorous synapse’. We propose that the amastigote flagellum acts as a sensory organelle with potentially important functions in the intracellular stage of the Leishmania life-cycle.

270/B217
The Hydrolethalus Syndrome Protein HYLS-1 Links Core Centriole Structure to Cilia Formation.
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Centrioles perform two major functions in eukaryotic cells: 1) they recruit pericentriolar material proteins to form centrosomes that organize the cellular microtubule cytoskeleton, and 2) they template cilia, microtubule-based cellular projections with sensory and motile roles. Identifying the molecular mechanisms underlying the dual functionality of centrioles remains an important current challenge. Here we show that HYLS-1, a protein mutated in hydrolethalus syndrome, a perinatal lethal human developmental disorder, is a centriolar protein required for ciliogenesis. HYLS-1 is stably incorporated into centrioles during their assembly in a manner dependent on SAS-4, a core centriolar protein with which it directly interacts. Unlike SAS-4, HYLS-1 is dispensable for centriole duplication and centrosome function in cell division. Instead, HYLS-1 is specifically required for cilia formation in C. elegans and Xenopus. The hydrolethalus-associated missense mutation impairs HYLS-1 function in ciliogenesis, indicating that hydrolethalus syndrome is a severe ciliopathy. Our results further show that HYLS-1 does not contribute to the intraflagellar transport (IFT) -dependent extension of the ciliary axoneme, but is required for the earlier, IFT-independent, step of apical migration and/or anchoring of centrioles at the plasma membrane. Thus, HYLS-1 is the first core centriolar protein not involved in centriole assembly or centrosome function in cell division. Instead, incorporation of HYLS-1 confers on centrioles the capacity to initiate ciliogenesis.

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EF Hand Mutations in a Tetrahymena Basal Body Protein, Cen1, Suggests a Possible Structure-Function Relationship.
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The basal body is a microtubule-based structure responsible for nucleating the cilium and anchoring it in place at the surface of the cell. Cilia are involved in a wide variety of important roles in metazoan cells, ranging from fluid flow to sensing the surrounding environment. A major question about the basal body is how are its molecular components involved in building, maintaining, and regulating the structure. A molecule that is conserved at basal bodies in many metazoans is a ~20kDa protein called centrin. Structurally, centrin consists of two independent domains tethered by a linker with each domain containing two EF hands, a Ca^{2+} binding motif. It has been shown that centrin is essential for proper basal body function; however, its molecular mechanisms and the specific role of its EF hands remains a mystery. We are using the ciliate protist Tetrahymena thermophila as a system to study basal bodies. Tetrahymena Cen1, which is closely related to the human Centrin2, is essential for the assembly and maintenance of basal bodies. We have mutated individual EF hands in Cen1 in order to gain a better understanding of its function. N-terminal EF hand mutations in Cen1 results in basal body orientation defects that occur only during the assembly of new basal bodies. Similar mutations to the EF hands of the C-terminal domain reduces the localization efficiency of Cen1, and these cells exhibit significant defects in basal body assembly and/or maintenance. Subsequent work shows that the C-terminal half of Cen1 is important for localization to basal bodies. These results raises the ideas that Ca^{2+} may be important for Cen1 function and that Cen1’s two domains have distinct functions, with the C-terminal domain being important for localization and the N-terminal domain regulating the protein’s function.

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A Screen for Chlamydomonas Insertional Mutants with Altered Deflagellation-Induced Gene Expression.
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Deflagellation of Chlamydomonas results in rapid and coordinate induction of hundreds of genes. The resulting new protein synthesis is required for regeneration of full-length flagella. Although Chlamydomonas is the preeminent model organism for the study of flagellar structure and function, the proteins required for deflagellation-stimulated gene induction are unknown. Chlamydomonas is an ideal organism for genetic analysis of flagella-specific gene induction. It
can be transformed at high frequency with dominant selectable markers and the introduced DNA integrates throughout the genome, simultaneously disrupting and tagging genes at the sites of insertion. Following mutagenesis, haploid genetics allows straightforward identification of recessive mutations causing a phenotype of interest. We have developed a reporter strain, LC8GLuc4, in which expression of a codon-optimized *Gaussia princeps* luciferase (Shao and Bock, 2008, *Curr Genet*) is driven by the dynein light chain LC8 promoter. Deflagellation of LC8GLuc4 results in an increase in luciferase activity similar to increases in expression of genes encoding flagellar proteins. Induction is detectable using a standard luciferase assay within 15 minutes after deflagellation and peaks at ~10- to 20-fold by 60 minutes. The ease of the luciferase assay makes high-throughput screening for insertional mutants in the deflagellation-stimulated gene-induction pathway feasible. In a pilot screen, we mutagenized LC8GLuc4 with a fragment of pHyg3 (Berthold et al., 2002, *Protist*). Among the hygromycin-resistant transformants, we identified a strain that lacks flagella and exhibits constitutive luciferase upregulation. Importantly, most aflagellate strains generated in the reporter background do not constitutively upregulate luciferase activity, indicating that upregulation is not simply a response to lack of flagella. We are currently expanding this pilot screen to identify mutants unable to normally induce gene expression following deflagellation.

**273/B220**

**Characterization of Putative Cilia Genes by High-Content Analysis.**

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Cilia are microtubule-based structures that protrude from the surface of the cell and are involved in a number of essential signaling pathways, yet much is still not known about all of the proteins that regulate cilia structure and function. We have sought to characterize 41 putative cilia genes of unknown function previously identified by proteomics and sequence analysis. We measured the effect on cilia of siRNA knockdown of these genes using: 1) a microscopy-based assay in 3T3 cells, measuring cilia number, cilia length, and hedgehog-dependent transport of endogenous Gli3 to the cilia tip; 2) a microscopy-based assay in IMCD3 cells, measuring the hedgehog-independent transport of HTR6-GFP to the cilia shaft; and 3) a Gli-luciferase reporter assay in S12 (C3H10T1/2) cells, measuring hedgehog signaling. 31 of the genes resulted in interesting cilia phenotypes upon siRNA knockdown, divided into five phenotypic classes: including loss of cilia, shorter cilia, longer cilia, and normal cilia with cargo transport and/or signaling defects. We will summarize results of the three assays on all of the cilia candidate genes, as well as an in depth analysis of at least one validated hit.

**274/B221**

**Functional Defects in Sperm from SPAG16L Disrupted Mice.**

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Sperm from mice homozygous for a disrupted *Spag16L* gene (KO sperm), which encodes a central apparatus protein, were examined to assess their flagellar motor functions. The beat frequency, bend curvature and shear angle development of live sperm carrying the defect were compared to those of age- and strain-matched control sperm. Live KO sperm exhibited much less bending of the flagellum during the beat. Shear angle analysis showed that the amount of microtubule sliding in the R-bend direction of the beat was selectively restricted. This suggests that there is limited activation of the dyneins on one side of the axoneme in the live cells. The KO sperm were also demembranated with Triton X-100 and examined for their response to free Ca2+ and for their ability to undergo dynein-driven microtubule sliding. The flagellar curvature response to Ca2+ is significantly (p < 0.001) reduced in the KO sperm. The Ca2+ response requires the activation of the dyneins on outer doublets 1, 2, 3 and 4. These are the same dyneins required for R-bend formation. In axonemes prepared to disintegrate by microtubule sliding, we observed little or no extrusion of doublets 1 and 2, which is consistent with a reduced activity of their dyneins. In
addition, the passive flagella inhibited by sodium metavanadate to disable the dyneins were examined for mechanical abnormalities. We discovered that the midpiece region of the KO sperm is more rigid than that of wild-type sperm. The deficit we observed in motor function, together with the increased rigidity of the midpiece region can account for the motility characteristics of the SPAG16L disrupted sperm. The lack of a normal response to Ca$^{2+}$ would render these sperm incapable of hyperactivation, which might explain the infertility associated with this defect. Our findings implicate an important role for the central apparatus in Ca$^{2+}$-induced hyperactivation. Supported by N.S.F. grant MCB-0516181 (CBL) and N.I.H. grant HD037416 (JFS).

275/B222  
**Developmental Regulation of the Ciliary Proteome in Sea Urchin.**  

The sea urchin embryo has long been a model of choice for studying the formation and function of cilia. In most urchin species, ciliogenesis occurs on all cells just prior to embryo hatching and is regulated thereafter in a tissue-specific manner to generate cilia with different lengths and behaviors. Probing the genome of the purple sea urchin Strongylocentrotus purpuratus using the flagellar proteomes from human and Chlamydomonas, we studied how the urchin regulates tissue-specific differentiation of cilia in development. Genes representing all ciliary protein classes including the flagellar-associated proteins (FAPs) in the Chlamydomonas Flagellar Proteome, were identified in the S. purpuratus genome by reciprocal BLAST to generate a draft sea urchin ciliome. Analysis of S. purpuratus transcriptome data from sequential developmental stages revealed that the majority of putative ciliary genes exhibit four general temporal expression patterns: predominantly zygotic, zygote-blastular, and early blastular (prior to or during initial ciliogenesis), and larval (coinciding with formation of ciliary subtypes). Few putative ciliary genes exhibit peak expression during the gastrula stages. Some groups of genes whose products presumably function together, such as IFT genes, are expressed prior to or during ciliogenesis but have variable peak expression times. Most FAPs that show modulated expression exhibit a discrete peak at the onset of ciliogenesis in the early blastula. Gene Ontology suggests that ciliary proteins with general metabolic functions exhibit peak expression early in development while many ciliary proteins with more specialized functions exhibit peak expression only after organogenesis. Genes whose products are central to specific processes, such as kinesin-2 family members that drive anterograde IFT, are being targeted by antisense morpholino knockdown to test their roles inferred by expression pattern and Gene Ontology analysis. Producing a draft ciliary proteome in the sea urchin, with its readily available transcriptome data, has allowed us to study how the ciliogenic program can be modulated during development to generate cilia with tissue-specific morphologies, motilities, and functions.

276/B223  
**Chlamydomonas Kinesin 13, a Member of the Microtubule-Depolymerizing Family of Kinesins Functions Both in Flagellar Assembly and Disassembly.**  
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Cilia and eukaryotic flagella are assembled and disassembled during cell cycle progression and upon environmental cues. Growth of the microtubule-based axoneme within the organelle requires assembly of microtubules at the tip of the flagellum, and axonemal disassembly depends on microtubule depolymerization at the tip. In *Chlamydomonas*, experimentally deflagellated cells immediately begin to regenerate new flagellar microtubules from tubulin precursor present in a pool in the cell body. Little is known about the molecular mechanisms that regulate microtubule dynamics during flagellar assembly and disassembly, nor is it known whether the cell body pool of tubulin is soluble or in the form of cell body microtubules. We have determined that CrKinesin13, a *Chlamydomonas* member of the kinesin13 family of microtubule depolymerizers, functions both during flagellar assembly and disassembly. Stimulation of a cell to assemble new
flagella triggers phosphorylation of CrKinesin13, whereas activation of flagellar shortening induces immediate transport of CrKinesin13 into the flagella from the cell body via intraflagellar transport (IFT). Cells depleted of CrKinesin13 by RNAi produce short flagella and are inhibited in flagellar shortening. Moreover, after deflagellation the CrKinesin13-depleted cells exhibit a 2 hr lag in regeneration, and assembly is blocked completely when protein synthesis is blocked. We propose that, in addition to its role during flagellar shortening to disassemble microtubules at the flagellar tip, CrKinesin13 functions in the cell body to mobilize tubulin from assembled microtubules for transport into the flagella. This work is partly supported by the National Natural Science Foundation of China (Grants 30671090, 30830057, and 30771084), National Institutes of Health (US) Grant GM25661, and the National Basic Research Program of China (Grant 2007CB914401).

277/B224
Heterotrimeric Kinesin-2 Is Essential for the Assembly and Functioning of Adult Olfactory Cilia in Drosophila.
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Type-I sensory neurons in insects express cilia from the distal ends of their dendrites, which contain the machinery for stimulus reception and transduction. Although diverse in structures and functions, basic organizations are similar in all these cilia. They contain either an axoneme, or, a set of doublet microtubule bearing proximal segment; and a membranous distal region consisting of singlet microtubules and/or actin filaments. Cooperative and partly redundant anterograde transport by the homodimeric Osm3/Klf17 class of motors and the heterotrimeric kinesin-2 were shown to be essential for the assembly and maintenance of sensory cilia in worms and mammals. However, only kinesin-2 subunits were found necessary and sufficient to maintain the chordotonal cilium in Drosophila, bearing a 9+0 axoneme. All Drosophila olfactory neurons also express elaborate sensory cilia containing both the axoneme bearing inner, and singlet microtubule containing outer segments, but the mechanism underlying their assembly and maintenance were unclear. We found that all the kinesin-2 subunits are enriched in the olfactory cilia in adult Drosophila antennae. In addition, mutations in the kinesin-2 motor subunit gene Klp64D significantly reduced the odor receptions and enhanced reception thresholds for a variety of odors at the adult antenna. Ultrastructure analysis of multiple mutant alleles further showed that loss of KLP64D reduced membranous branches and microtubule localizations in the outer segments, whereas the loss of the accessory subunit DmKAP eliminated the entire cilia, indicating a complex and differential roles of kinesin-2 subunits in adult olfactory cilia.

278/B225
The CSC Is Required for Complete Radial Spoke Assembly and Wild-Type Ciliary Motility.
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We previously identified a CaM and spoke associated complex (the CSC) and demonstrated a role for this complex in regulating dynein-driven microtubule sliding (Dymek and Smith, 2007). One CSC member, CaM-IP2, interacts with RSP3, an AKAP that links the spoke to the doublet microtubule. Based on this association, we hypothesized that interactions between CaM-IP2 and RSP3 play a role in spoke assembly and/or modulating motility. The CSC assembles onto the axoneme in the radial spokeless mutant pf14. Therefore, assembly of the CSC does not require the radial spokes. What is not known is, whether spoke assembly requires the CSC. No mutations have been identified that correspond to any of the three CSC components. Therefore, we used an artificial microRNA approach to reduce expression of CSC components (Zhao et al., 2008; Molner et al. 2009). Using constructs designed to silence expression of either CaM-IP2 or CaM-IP3, we obtained two mutants with reduced expression of these components. In both mutants, CaM-IP2 and -IP3 are lacking or severely reduced in isolated flagella, and immunoprecipitation with anti-CaM antibodies fails to precipitate any members of the CSC. Western blots of flagella using antibodies directed against radial spoke components suggest that a fraction of radial spokes failed to assemble in these mutants. Images of mutant axonemes using
thin section electron microscopy were consistent with results from western blots and revealed an unusual pattern of altered spoke assembly. In analyses of motility in these mutants we discovered that while swimming speed was reduced by 25-30% compared to wild-type, beat frequency was only slightly reduced. This reduction in beat frequency was not sufficient to account for the reduction in swimming speed. Upon closer inspection of flagellar beating by high speed video microscopy, we discovered that the flagella of these mutants often lost coordination, beating out of sync with one another. This lack of coordination resulted in frequent pauses rather than more constant trajectories. Our results strongly suggest that the CSC is associated with only a subset of radial spokes and that this association is required for complete spoke assembly and wild-type motility.

279/B226
**Calcium Induces Transcription/Translation-Independent Ciliogenesis.**
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Cilia and flagella play critical roles in the development and function of vital organs. To elucidate the fundamental mechanisms controlling their generation, we take advantage of a usually aflagellate *Chlamydomonas* strain, *fla14*. This strain has defective cytoplasmic dynein motors and can only grow half-length flagella at best due to the absence of retrograde intraflagellar transport (Pazour et al. 1998). Flagella generate in 0.5 - 2 hours following suspension in distilled water or 10 mM HEPES buffer and exposure to light, a common practice to encourage flagella generation. Contrarily, aflagellate cells defective in axonemal complexes can generate flagella in 10 minutes after the suspension. Culture media can promote growth only when diluted at least four-fold. Addition of calcium is not required. The translation inhibitor cycloheximide has no effect on the process, while 10 mM KCl that depolarizes cells accelerates growth. In contrast, growth is inhibited by 50 μM EGTA, 10 mM NaCl, repeated suspensions, and Verapamil, an L-type voltage-gated calcium channel blocker. Furthermore, cells harvested from spent media cannot be induced to generate flagella. These results indicate that ciliogenesis is controlled by multiple pathways including calcium signaling. Hypo-osmolarity amplifies the light-induced depolarization, leading to the release and re-entry of calcium which signals flagellar generation downstream from transcription and translation.

280/B227
**Structural Model of Microtubule Lateral Deformation Based on Studies of Multiple Types of Microtubules.**
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Microtubules are hollow cylinders formed by tubulin protofilaments. Microtubules are often exposed to mechanical stress, for example from motor-induced forces or passive bending under external forces. Rather than being rigid cylinders, microtubules are flexible and able to deform, which may be an essential property for them to tolerate environmental stress. Lateral deformation of microtubules has been identified in our cryo-tomography study of microtubule doublets, which are the major structure component of the axoneme. The molecular architecture of the doublet and the axoneme obtained by tomography has shed light on the mechanical properties of cilia/flagella. One structural feature revealed is that the microtubule in the intact doublet shows an oval shape in cross-section (Sui and Downing, Nature 2006). This distortion represents flexibility of the interprotofilament interactions in the microtubule and presumably results from mechanical stress that arises from binding of microtubule-associated proteins. It may also contribute to the anisotropic bending moment of doublets. Here we propose a structural model for lateral deformation based on studies of multiple types of microtubules. While most microtubules are found to contain 13 protofilaments, the interactions between adjacent protofilaments is sufficiently flexible to allow the number of protofilaments in a single microtubule to vary from 9 to 16. We used cryo-electron microscopy to study structures of microtubules composed of different numbers of protofilaments. Resultant density maps have reached a resolution sufficient to clearly reveal the tubulin secondary structure and enabled construction of pseudo-atomic models for multiple types of
microtubules. In these models, the structures of the protofilaments do not display any noticeable differences, other than that the angle between neighboring protofilaments changes with the number of protofilaments. Inter-protofilament interactions involve the M and H1-S2 loops. The loop-loop interactions offer structural flexibility and are therefore proposed to be the major contributor for the lateral deformation of the microtubule in our model.

281/B228
Defining Functional Domains in PF6, a Component of the Central Apparatus C1a Projection and Required for Ciliary/Flagellar Motility.
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The c1a projection of the flagellar central apparatus is critical for normal motility, as evidenced by Chlamydomonas pf6 mutants, which lack c1a and have twitchy flagella unable to generate productive movement. We have previously shown that PF6 forms a complex with C1a-86, C1a-34, C1a-32, C1a-18, and calmodulin (CaM); this entire complex fails to assemble in pf6 flagella (Wargo, et al., 2005). Based on this defect, PF6 (2301 amino acids) most likely serves as a scaffold or adaptor for complex assembly onto the C1 central tubule. Since no known mutants exist for other members of the PF6 complex, their specific contributions to motility are unknown. Presumably, CaM plays a role in calcium regulation of motility. As a first approach to generating mutants which fail to assemble specific members of the PF6 complex, as well as to define functional domains within PF6, we have created deletion constructs of PF6 and transformed these into pf6 mutants. Transformants expressing PF6 lacking either amino acids 68-752 or amino acids 854-1821 assemble truncated PF6 protein as well as all c1a projection components into their flagella. Motility is also restored to pf6 cells transformed with either construct. However, swimming velocity is decreased to one-half that of wild-type. This decrease is primarily due to a 50% reduction in beat frequency, although subtle waveform defects may also exist. These results suggest that the amino two-thirds of PF6 may interact with axonemal proteins other than PF6 complex components, and that these interactions are evidently critical for wild-type motility. In contrast, no motile cells are recovered when pf6 is transformed with a construct that produces PF6 lacking C-terminal amino acids 1459-2301. A smaller C-terminal deletion (1863-2230), which disrupts a basic domain and conserved ASH domain (Ponting, 2006) restores motility. Swimming velocity and beat frequency of these cells is slightly reduced compared to wild-type. Biochemical analyses indicate that the associations of C1a-86 and CaM with this smaller C-terminal deletion are unstable. These results indicate that the carboxyl one-third of PF6 is required for assembly onto C1 as well as for stable interaction with PF6 complex members.

282/B229
3D Views of the Dynein Regulatory Complex by Cryo-Electron Tomography.
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Cilia and flagella are highly conserved eukaryotic organelles with motile or sensory functions. In humans, many cell types assemble cilia, and they are crucial for the proper functioning of several organs. Ciliary defects are thus associated with a number of diseases, such as polycystic kidney disease or primary ciliary dyskinesia [1]. Most cilia share a common core-structure, the axoneme, which consists of nine doublet microtubules surrounding a central pair of singlet microtubules [2]. Motility is generated by the orchestrated activity of thousands of dynein motors that drive inter-doublet sliding. Structures that restrict sliding, such as the nexin-links that connect neighboring doublets, transform inter-doublet sliding into axonemal bending [3, 4]. The dynein regulatory complex (DRC) has a key role in controlling dynein activity and thus axonemal movement [5, 6]. Seven DRC components have been identified and characterized biochemically [5]. However, few details about the structural organization of the DRC are known. We analyzed rapidly frozen Chlamydomonas axonomes by cryo-electron tomography followed by image processing to gain new insights into the 3D structure and molecular organization of the DRC in situ. By correlating published biochemical data with our tomograms from the wildtype and five drc-mutant strains we

283/B230
A Conformational Switch for Outer Arm Dynein Motor Function.
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Based on the experimental manipulation of mutant Chlamydomonas flagella lacking various axonemal substructures, Hayashibe et al (CMC 37, 232 [1997]) proposed the existence of two mechanosensory systems that respond to alterations in the mechanical state of the axoneme and allow for propagation of a flagellar bend along the axonemal length. One sensor appears to involve the central pair complex and impinges on the inner dynein arms, whereas a second distinct system was proposed to control outer arm function. The LC1 light chain within outer arm dynein associates with the motor domain of the gamma dynein heavy chain (HC), and crosslinking experiments, blot overlays and direct binding assays also indicate that it associates directly with tubulin. Based on both geometric constraints derived from the NMR structural ensemble (PDB 1M9L) and immunogold electron microscopy, LC1 appears to tether the HC motor unit to the A-tubule of the outer doublets. Thus, this dynein HC is bound to the same microtubule by both its N-terminal region and motor domain. We expressed mutant forms of LC1 (designed based on our structural data) in a wildtype background and found that they had dramatic dominant negative effects on cell swimming velocity but yielded only minor alterations in beat frequency. To further assess these observations, we determined the propulsive force generated under varying viscous load. We observed that these strains had a low force output similar to outer arm-less mutants under low viscous load but that this increased towards wildtype levels as load increased (and beat frequency decreased); this response essentially phenocopied the response observed for a mutant strain (oda2-t) lacking the gamma HC motor domain. Furthermore, high speed imaging revealed that the flagella of strains expressing mutant LC1 proteins almost always beat completely out-of-phase leading to a rolling forward motion. Furthermore, the flagella randomly stalled for a few msec at or near the power/recovery stroke transition point. We propose that the gamma HC/LC1/microtubule ternary complex acts as a conformational switch to control outer arm function.

284/B231
DPY-30, a Versatile Docking Domain, Targets Structural and Signaling Modules for Integrated Control of Flagellar Beating.
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Numerous examples demonstrate that signal transduction pathways are localized to specific micro-compartments to ensure strict spatial or temporal regulation. A cardinal example is the targeting of cAMP-dependent Protein Kinase (PKA) via the RIIa domain in the regulatory subunit RII. Curiously, existing database contains nearly 400 molecules with either RIIa domain or a RIIa-like, DPY-30 domain. The additional sequences in these molecules are often irrelevant to PKA but rather related to nucleotide metabolism or calcium signaling. Particularly, many have short and inconspicuous sequences. Examples include DPY-30, a subunit in various chromosome modification complexes and mammalian homologues of two Chlamydomonas radial spoke proteins, RSP2, that is critical for the assembly of the spokehead module and RSP23, a nucleoside diphosphate kinase. To elucidate the role of DPY-30 domain, we focus on RSP2 and RSP23. Both contain calmodulin-binding domains, yet the putative calcium-sensing elements are absent in their homologues. We found that RSP2 mutants defective in calmodulin-binding are
normal in motility and phototaxis but display light-induced irregular trajectories, revealing a novel pathway that inhibits phototaxis amid conflicting light and barrier. On the other hand, RSP2 mutants lacking DPY-30 domain are paralyzed although all spoke proteins are assembled. Crucially, the polypeptide lacking DPY-30 domain and spokehead proteins dissociate after salt extraction. In contrast, the molecules containing the DPY-30 domain are always stable. These findings strongly suggest that the DPY-30 domain docks structural, enzymatic and calcium-sensing modules to a critical location in the radial spoke to integrate assembly and local nucleotide metabolism with a calcium/calmodulin-dependent signaling pathway special to phototrophic *Chlamydomonas*. This result reveals the extraordinary versatility of RIIa-like domains that localize various critical functional moieties, often in different combinations tailored to the unique needs of individual organisms.

**285/B232**

**Outer Arm Dynein Is Required for Gliding Motility in Planaria.**
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Planarians are free living, non-parasitic flatworms and a model system for stem cell biology and regeneration. *Schmidtea mediterranea* is a species of planarian (Class: Tubellaria) with a well-defined monostratified ciliated epithelium. The current notion is that planarians, like other invertebrates (e.g. Mollusks), use their cilia for movement; however, the requirement of ciliary motility for planarian locomotion has not been tested directly. Planarian cilia are ubiquitously distributed on the ventral surface and are motile; beating at ~20 Hz using a highly asymmetric waveform. We studied the role of cilia in planarian physiology using RNAi in order to target conserved genes that are essential for cilia biogenesis (IFT88) as well as outer arm dynein assembly (IC1 and IC2) and regulation (LC1). We found that when the normal function of cilia was disrupted, the flatworms moved significantly more slowly than controls fed with an empty vector. More importantly, in the absence of normal ciliary function, planarians switched their mode of movement from a smooth gliding-type of locomotion to a crawl, which was powered by peristaltic muscle contractions. This behavior was observed regardless of whether the flatworms 1) completely failed to assemble cilia, 2) had normal length cilia lacking outer dynein arms, or 3) were merely missing a key regulatory component of the outer arm dynein complex. Our results suggest that planarian motility consists of two independent components; namely ciliary beating and muscular activity. In planarians normal cilia activity is required for gliding locomotion but not for movement in general. These observations also raise intriguing questions regarding the mechanism by which planarians are able to coordinate ciliary motility and muscular activity in order to control their movement.

**286/B233**

**Identification and Characterization of the Major Tubulin Acetyltransferase.**
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Since its discovery more than 25 years ago, the acetylation of alpha tubulin at lysine 40 has remained the marker of choice for stable microtubules in general and cilia and flagella in particular. Yet, the functional consequence of tubulin acetylation has remained a major unknown. While it is generally believed that acetylation is a mere consequence rather than a causative factor of microtubule stabilization, recent evidence have pointed to a role for microtubule acetylation in the regulation of neurogenesis. Still, a major obstacle in our progress towards a functional understanding of tubulin acetylation is that the tubulin acetyltransferase remains unidentified. Recently, we discovered that one of the BBSome subunits is required for tubulin acetylation in cells and we proposed that the BBSome may contact and activate the tubulin acetyltransferase. Here, we find that Arl6<sup>GTP</sup> affinity chromatography of retinal extracts recovers large quantities of the BBSome and trace amounts of an uncharacterized protein that we have named TACT1. Surprisingly, structure-based alignments reveal that TACT1 harbors cryptic homologies to the GNAT clade of acetyltransferases and genome mining shows that TACT1 is
universally conserved among ciliated organisms. Thus, we hypothesized that TACT1 may constitute the long sought tubulin acetyltransferase. Strikingly, bacterially expressed TACT1 efficiently transfers the radioactively labeled acetyl group from acetylCoA onto tubulin. Further analysis of the reaction product demonstrate that Lysine 40 is a major acceptor site for the TACT1 enzyme. Furthermore, TACT1 exhibits remarkable substrate specificity as it is unable to acetylate Histone H3/H4 in vitro. In vivo, overexpressing TACT1 is sufficient to induce massive tubulin acetylation in RPE, IMCD3, Hela and in particular in Ptk2 cells that entirely lack tubulin acetylation. at the same time, overexpression of a catalytically dead mutant of TACT1 does not result in any changes in acetylation levels in cells. Finally, depletion of TACT1 in RPE cells results in a significant decrease in tubulin acetylation. Thus we conclude that TACT1 is the major Tubulin AcetylTransferase in mammalian cells.

287/B234
The Role of Microtubule Posttranslational Modifications in Cilia Formation and Function.
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One of the most striking discoveries in the past decade is that a large number of a priori unrelated human diseases (including certain forms of polycystic disorders, cognitive defects, obesity and retinal degeneration) result from defects in the structure and function of a single cell organelle: the cilium. The cilium is a microtubule (MT)-based organelle that projects from the surface of most eukaryotic cells to perform two major biological roles, sensory transduction and cell motility, which explains why cilia-related disorders can affect diverse organ systems. The proper functioning of a cilium depends on a variety of MT-associated proteins (MAPs) and molecular motors that interact with its MT core structure, the axoneme. The essential question of how the specific interactions between MTs and their interacting proteins remains not completely understood. One common feature of ciliary MTs is that they are strongly modified by a range of posttranslational modifications, including glutamylation and glycylation. Since these modifications have been suggested to regulate the interactions between MTs and MAPs/motors, it is generally considered that they are important regulators of cilia assembly and function. However, the knowledge on the functions of tubulin glutamylation and glycylation is still restricted since the modifying enzymes have only recently been discovered. Both, tubulin glutamylases and glycylases are member of the Tubulin Tyrosine Ligase Like (TTLL) family. Each modifying enzyme has particular specificities, allowing for the generation of complex modification patterns. To address their potential role, we have first characterised the distribution of MT glutamylation and glycylation in axonemes of mouse ependymal cells and photoreceptors, as a model for motile and sensory cilia respectively. Next, to study the impact of MT modifications patterns on ciliary integrity and function, we have established a cell model for culturing ependymal cells. Our first results show that overexpression or suppression of certain MT modifying enzymes results in a reduced number of cilia, which suggests that glutamylation and glycylation may be essential for cilia assembly.

288/B235
Essential Role of Tubulin Polyglutamylation in Asymmetric Cilia Bending and Beating.
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Mammalian respiratory cilia generate unidirectional flow by beating asymmetrically. What is essential for the asymmetric beating as well as how the asymmetric beating is achieved are central questions in cell biology. Tubulin constructing ciliary or flagellar axoneme undergoes a unique post-translational modification, polyglutamylation. Polyglutamylation occurs on glutamate residues in the carboxy terminal of tubulin. Under the modification, a long peptide chain composed of multiple glutamate residues (>10 glutamates) are attached to gamma-carboxyl group of glutamate residue in the tubulin carboxy terminal. Physiological significance of tubulin
polyglutamylation in ciliary function is also poorly understood. We in this work examine whether tubulin polyglutamylation is required for the asymmetric ciliary beating. We generated a knockout mouse that lacked a polyglutamylation-performing enzyme, TTLL1, and investigated alterations in structure and motility of tracheal cilia of TTLL1-knockout mouse. Axonemes of tracheal cilia of TTLL1-knockout mice grossly lost polyglutamylated tubulins. Surprisingly, axonemes isolated from TTLL1-deficient mice were straighter compared to wild-type axonemes, which were well bent. Similar a result was obtained under the presence of low concentration of ATP, where axonemes became a relaxed state from a rigor state. This indicates that the axoneme bending observed in wild type occurs intrinsically in axoneme and that tubulin polyglutamylation is required for the axoneme-intrinsic bending. In parallel with this, the axoneme isolated from TTLL1-knockout mouse lost beating asymmetry, i.e. it was difficult to distinguish between effective and recovery strokes. The loss of beating asymmetry was also observed in cilia motility of TTLL1-knockout mouse trachea. In agreement with this finding, the mucociliary function is drastically reduced in TTLL1-knockout mice. Finally, TTLL1-knockout mice showed a severe respiratory phenotype; the mice frequently coughed and sneezed. In conclusion, tubulin polyglutamylation is essential for structural and functional asymmetry of respiratory cilia.

289/B236
Tubulin Polyglutamylation Is Regulated by TTLL-4 Polyglutamylase through Ca²⁺-MAPK Signaling Pathway.
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The primary cilium is a microtubule-based organelle that is significant for sensory signal transduction. Sensory channels and receptors are transported and condensed on the sensory cilia through intraflagellar transport (IFT). Polyglutamylation is a post-translational modification that adds multiple glutamates to specific glutamate residue within tubulins and is proposed to be important for several microtubule-related processes. Polyglutamylation is accomplished by TTLL family protein polyglutamylases, but their functions In Vivo and the regulatory mechanisms are not well characterized. In this study, we have tried genetical approach to elucidate these questions using C.elegans. Immunohistochemistry with the polyglutalylated tubulin-specific antibody revealed that the signals are only detected in the cilia of sensory neurons. C.elegans has six ttll genes—ttll-4, -5, -9, -11, -12 and -15. In the sensory neurons, TTLL-9 is distributed in a whole sensory neuron. On the other hand, TTLL-4 and TTLL-11 are complementarily localized in cilia and axons, respectively. Analyses for mutant strains for each gene have revealed that ttll-4 is the only gene that regulated polyglutamylation levels in sensory cilia. The ttll-4 mutant worms completely lost the polyglutamylation signals, whose sensory neurons show abnormal ciliary structure and also IFT defects. The ttll-4 mutants move normally but they show some sensory defects, such as chemotaxis for NaCl and nose-touch response, which is sensed by amphid sensory neurons. Finally, we analyzed the regulatory mechanism of ttll-4. Several mutant strains of Ca²⁺-MAPK pathway, most of which have chemosensory defects, show the reduction of polyglutamylation, suggesting that ttll-4 is regulated by conventional environmental signals. Notably, excess artificial signals such as vibration and heat-stress also affect the level of polyglutamylation. According to the experimental results above, we can conclude that TTLL-4 is a key polyglutamylase that controls IFT for the appropriate sensory transduction and is regulated by environmental signals through Ca²⁺-MAPK signaling pathway.

Techniques, Regulation, Structural Basis (290 – 310)

290/B237
Engineered Allosteric Regulation of Kinases in Living Cells.
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Manipulation of protein activity in living cells is an extremely valuable tool, but for many specific proteins is not available or suffers from serious limitations. We describe here a new approach for selective allosteric regulation of a single domain within a multidomain protein, and demonstrate a broadly applicable method to activate kinases in living cells with high specificity and within minutes. A new small domain, named iFKBP, was developed for insertion in the middle of protein structures, where it acts as an allosteric switch responding to the small membrane-permeable molecule rapamycin. Introduction of iFKBP into a catalytic domain of focal adhesion kinase (FAK) enabled specific regulation of its kinase activity without affecting other properties of FAK. Using this new tool, we provide the first direct demonstration of FAK-mediated activation of Src in live cells. Furthermore, we show that that FAK stimulates formation of large dorsal protrusions via activation of Src, a new property for FAK, implying a mechanism for its involvement in tumorigenesis. Computational studies reveal a potential mechanism for iFKBP-mediated regulation and, together with generation of rapamycin-regulated Src, demonstrate general applicability of the method.

291/B238
Genetically-Encoded, Reversible Photoactivation of Rac and Cdc42 in Living Cells Illuminating the Coordination of Rho GTPases in Cell Motility.
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For many highly dynamic cell behaviors such as cell migration, signaling events occur transiently at specific locations, with subsecond and submicron precision. Study of such rapid dynamics has been hindered by our inability to manipulate protein activities with precise spatio-temporal control in living cells. Recently we engineered a genetically-encoded photoactivatable Rac1 (PA-Rac1) using a light-responsive LOV (light, oxygen or voltage) domain from an oat phototropin protein. A LOV domain fused to constitutively active Rac1 sterically blocked the active site of Rac1 until illumination at specific wavelengths induced a LOV conformational change, releasing inhibition. In living cells, PA-Rac1 enabled reversible and repeatable control of Rac1 activation at precise times and subcellular locations. This was used to specify the direction and extent of cell locomotion. Combining PA-Rac1 with a biosensor of RhoA activation demonstrated localized control of Rac-Rho inhibition. We have also extended the photoactivation technology to dominant negative Rac1, via a related design, to the structurally similar small GTPase Cdc42. These technical developments pave the way to study the coordination between different GTPases during cell migration.

292/B239
Opposing Pathways Regulated by Rac1 and RhoC at the Leading Edge of MTLn3 Carcinoma Cells.
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p21 Rho family of small GTPases are involved in cell motility and migration. Preliminary results have provided evidence that overexpression of RhoC in the MTLn3 breast carcinoma cell line downregulates Rac1, but not Cdc42. While similar antagonism has been reported between RhoA and Rac1, such a mechanism between RhoC and Rac1 has not been directly observed. To study the relationship between RhoC and Rac1 during cell protrusion, we developed a novel, genetically-encoded fluorescent Rac1 biosensor, based on a single-chain design. Our design maintains the correct C-terminal lipid modification of full-length Rac1, enabling proper interaction with upstream regulators, including GEFs, GAPs, and GDIs. Because of the parallel amino to carboxy terminal directionality of both the binding domain and the Rac1 molecule, we could not simply exchange the GTPase and binding domain from the previous-generation RhoA single-chain biosensor (Pertz, et. al. 2006 Nature). We constructed this new single-chain biosensor by
modifying the placement of the binding domain in relation to the first fluorescent protein, to achieve the proper orientation for optimal interaction between the GTPase and its binding domain. By using this design, we were able to maintain a 1:1 donor to acceptor FRET ratio, which is an improvement over the previously designed Rac1 biosensor based on a bi-molecular design. This design should be generalizable to most GTPase Binding-domain pair with a similar parallel orientation. We used this novel single-chain biosensor, together with a previously-developed single-chain RhoC biosensor to investigate further the balance between these two molecules in the control of cancer cell motility. These biosensors allow visualization of the spatial and temporal activation patterns of Rac1 and RhoC simultaneously in real time in breast carcinoma cells. This work shows the interactions and spatiotemporal dynamics of the Rho family GTPase proteins.

293/B240
Screening of Compounds Modulating ARP2/3 Activity for Keratocyte Motility Effects Using a Cell Sheet Assay.
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Fish keratocytes used in cell motility studies are typically cultured in explant cultures for 24 - 48 hours to form cell sheets which are then replated before use in cell motility assays. In order to form the initial cell sheet, the cells associated with the scale must initially adhere to the culture surface and then the keratocytes migrate from the scale to form a cell sheet. We have used keratocyte cell sheet assay to rapidly screen for motility effects of compounds affecting the activity of the ARP2/3 complex. The percentage of scales with cell sheets is used as a measure of cell adhesion. The area of the cell sheet can be used to assess cell migration and spreading. Preliminary data indicates that wiskostatin, a cell permeable inhibitor of WASP, significantly inhibits cell sheet growth at 1 uM concentration. Although more data needs to be gathered, there is an apparent increase in the percentage of scales with cell sheets and a possible dose-response when wiskostatin dose is increased. These data suggest that measurements on keratocyte cell sheets in explant cultures may provide a rapid screen to identify compounds that effect cell motility.

294/B241
The Use of Laser Tweezers and the Mitochondrial Membrane Proton-Detecting Dye, DiOC(3), to Measure Sperm Motility.
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PURPOSE: The purpose of this study is to investigate how the mitochondrial membrane potential affects sperm motility using laser tweezers and the fluorescent probe, DiOC6(3) [Invitrogen dye D-273]. METHODS: A 1064 nm Nd:YVO4 continuous wave laser was used to trap motile sperm at a power of about 450 mW into a focused trap spot. Using customized tracking software, the curvilinear velocity (VCL) and the escape force (Pesc) from the laser tweezers were measured. Human sperm were labeled with the fluorescent dye DiOC6(3) as way to measure membrane potential in the sperm midpiece. The VCL was measured prior to, and after, sperm trapping. The fluorescence was measured before and during trapping. Fluorescence intensity of the DiOC6(3)-treated sperm were compared to untreated sperm. Controls were also performed to verify that the DiOC6(3), itself, does not affect sperm motility. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used to inhibit mitochondrial respiraion as measured by a loss of DiOC6(3) fluorescence. RESULTS: The results demonstrate that DiOC6(3) has no effect on the VCL and Pesc of the sperm even though there was a marked reduction in dye fluorescence when the CCCP was applied. Non DiOC6(3)-treated sperm had a VCL of 79.3µm/sec (n = 72), while DiOC6(3)-treated sperm had an average of 84.5 µm/sec (n = 232). The statistical difference between the two groups was p = .294 using the Wilcoxon rank-sum test. CCCP + DiOC6(3) labeled sperm had a VCL of 83.3 µm/sec, which was not statistically different from either of the
previous two groups of sperm. DiOC6(3)-labeled sperm exhibited a fluorescence (pixel intensity) of 96.3, unlabeled sperm 80.0, and CCCP-DiOC6(3) treated sperm 79.85. The unlabeled and CCCP-DiOC6(3) sperm appear similar to the background intensity. Despite the statistically significant difference between the DiOC6(3) fluorescent sperm, CCCP-DiOC6(3) sperm, and non-DiOC6(3)-treated sperm, the motility parameters are statistically equal for all groups. These results suggest that the primary driving energy source for motility in human sperm is not from aerobic mitochondrial respiration. The results for dog and mice sperm will be compared to human sperm.

295/B242

In Silico Reconstitution of Actin-Based Motility.

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Eukaryotic cells assemble viscoelastic networks of crosslinked actin filaments to control their shape, mechanical properties, and motility. One important class of actin network is nucleated by the Arp2/3 complex and drives both membrane protrusion at the leading edge of motile cells and intracellular motility of pathogens such as Listeria monocytogenes. These networks can be reconstituted in vitro from purified components to drive the motility of spherical micron-sized beads. An Elastic Gel Model has been successful in explaining how these networks break symmetry, but how they produce directed motile force has been less clear. We have combined numerical simulations with in vitro experiments to reconstitute the behavior of these motile actin networks in silico using an Accumulative Particle-Spring (APS) model that builds on the Elastic Gel Model, and demonstrates simple intuitive mechanisms for both symmetry breaking and sustained motility. The APS model explains observed transitions between smooth and pulsatile motion as well as subtle variations in network architecture caused by differences in geometry and conditions. Our findings also explain sideways symmetry breaking and motility of elongated beads and show that elastic recoil, though important for symmetry breaking and pulsatile motion, is not necessary for smooth directional motility. The APS model demonstrates how a small number of viscoelastic network parameters and construction rules suffice to recapture the complex behavior of motile actin networks. The fact that the model not only mirrors our in vitro observations, but also makes novel predictions that we confirm by experiment, suggests that the model captures much of the essence of actin-based motility in this system.

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Force Transmission from Actin Network to the Substrate in Migrating Cells.

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During cell migration, forces generated by actin cytoskeleton are transmitted through adhesion complexes to the substrate. To investigate the mechanism of force generation and transmission, we analyzed the relationship between the velocity of actin network and traction stress applied to the substrate using a simple model of persistently migrating fish epidermal keratocytes. Local forward stress at the back of the cell was largely proportional to the local actin velocity. Similar relationship between actin velocity and stress was observed at the sides of the cell, but the stress/velocity curve was steeper than at the back. In contrast, backward propulsive stress at the cell front exhibited significant velocity-independent component. These results suggested that the mechanisms of conversion of actin dynamics into the traction stress were different in different cell regions: back and sides of the cell were characterized by frictional slippage, while at the front actin network gripped to the substrate. Analysis of substrate stress and cell motion in the presence of inhibitors of actin/myosin system cytochalasin D and blebbistatin indicated that cell translocation could be driven by two different processes: actomyosin contraction, and actin assembly, the former associated with significantly larger traction stress than the latter.
297/B244  
**Turnover Dynamics of Diffuse Actin and Regulators at the Leading Edge.**  
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The lamellipodium at the leading edge of motile cells is a dynamic structure consisting of a dense network of branched actin filaments. These actin filaments polymerize in a region close to the leading edge and undergo retrograde flow towards the main body of the cell. A large number of experimental techniques have been used to monitor the structural aspects of actin networks at the leading edge. However, the role of concentration gradients and local heterogeneity of soluble actin, actin oligomers, and actin regulators in the crowded filament network is little explored. We combined single molecule fluorescence microscopy with image analysis and modeling to quantify the role of diffuse actin species and their gradients in actin reorganization at the leading edge of motile cells. Actin, capping protein, and Arp 2/3 complex, were marked with fluorescent probes at low concentrations and imaged at high spatiotemporal resolution in XTC fibroblasts. Particle tracking was used to mark the appearance and disappearance of bright spots that correspond to proteins becoming associated to, or dissociated from, the actin network. Image correlation analysis was used to quantify the motion of proteins in the cytoplasm. We developed conditional image correlation methods to study local dynamics prior to assembly and immediately following disassembly. From these data we create a map of the lamellipodium showing the dynamics of lamellipodium proteins and their turnover. Bounds on the fraction of actin that leaves the filament network as oligomers was determined by measuring the distribution of diffusion coefficients which correspond to different oligomer lengths. We used numerical simulations to model these turnover dynamics and to simulate FRAP experiments. These results help resolve apparently disparities in measurements found through FRAP and single molecule speckle microscopy.

298/B245  
**Automated Detection and Characterization of Pseudopodial Behavior during Amoeboid Motility.**  
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When distinguishing among strains of chemotaxing cells, it is common to compare aspects such as speed, chemotaxis index and persistence. However, amoeboid motion is a complex cellular process driven by highly-organized cytoskeletal dynamics involving alternating cycles of pseudopodial protrusions and retractions. A comparison of the pseudopodial dynamics may reveal subtle differences between some strains. Because of the highly variable stochastic fluctuations in the behavior of pseudopods, large data sets may be necessary, making manual detection, identification and quantification impractical. Here we present an automated method that automatically detects and characterizes pseudopodial behavior of cells. We illustrate the algorithm on movies of chemotactic *Dictyostelium* cells. The method uses skeletonization, a technique from morphological image processing to reduce a shape into a series of connected lines - the skeleton - which maintains prominent shape patterns similar to those perceived by human eyes. It involves a series of automatic algorithms including image segmentation, boundary smoothing, skeletonization and branch pruning, and takes into account the cell shape changes between successive frames to detect protrusion and retraction activities. In addition, the activities are clustered into different groups, each representing the protruding and retracting history of individual pseudopods. The algorithm tracks the state and angle dynamics for each pseudopod, and provides a number of characterizing quantities, such as the lifetime, splitting rate, state persistence, protrusion and retraction speeds, and angle changes. Additionally, more quantitative measurements can be acquired and analyzed according to the specific goals of study. No chemical markers are required for our method; however, the results can be correlated with other signals from the cell, such as fluorescent intensities, to facilitate the understanding of the relationships between motility and other cellular processes. In conclusion, the method provides a
powerful tool to investigate amoeboid motility by making it possible to capture the spatial and temporal dynamics as well as the stochastic features of the pseudopodial behavior.

299/B246
**Actin Tyr-53 Mutants Inhibit Chemotactic Signaling in Dictyostelium.**
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We recently published that phosphorylation of Tyr-53 of *Dictyostelium* actin, which occurs during development and when amoebae are subjected to stress, inhibits filament nucleation and elongation in vitro, and destabilizes filaments (PNAS 103:13694, 2006; PNAS 105:11748, 2008). To further study the function of Tyr-53 In Vivo and in vitro, we substituted Tyr-53 with Phe (Y/F), Ala (Y/A) or Glu (Y/E), fused a FLAG-tag at the N-terminus to facilitate purification, and expressed the mutant actins and FLAG-WT (Y/Y), as a control, in *Dictyostelium AX3* cells. The expressed actins accounted for about 25% of the total actin in cells. We found purified Y/Y and Y/F are very similar to WT actin but purified Y/A and Y/E polymerize more slowly, have a higher critical concentration, form abnormal, broken filaments, and have a higher Km for activation of myosin ATPase. In addition, co-polymers of 25% Y/A or Y/E and endogenous WT actin form shorter filaments than WT actin with a higher Km for activation of myosin. In Vivo studies showed that all expressed FLAG-actins co-localize with endogenous actin and that the mutant actins have no effect on growth in suspension, pinocytosis or phagocytosis. However, cells expressing Y/A or Y/E exhibit serious defects in chemotaxis, streaming and development. Cells expressing Y/A and Y/E have a 50% reduction in cAMP receptor number in both vegetative and developed cells, a 50% reduction in cAMP-induced ERK2 phosphorylation, a 75% reduction in cAMP-induced adenyl cyclase activation, and a 30-40% reduction in instant actin polymerization in response to cAMP. Our results suggest that the expression of Y53A and Y53E actin mutants affects actin structure and function In Vivo resulting in a global inhibition of cAMP-mediated signaling during *Dictyostelium* development.

300/B247
**Elucidation of Signaling Mechanism during Folic Acid Mediated Chemotaxis.**
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*Dictyostelium discoideum* chemotax towards folic acid during vegetative growth and to cAMP during aggregation and development. A number of signaling molecules are activated downstream of the heterotrimeric G proteins during cAMP-mediated chemotaxis including the Ras small G proteins, the TORC2 complex, PKBs, and the enzymes PI3K and PTEN which regulate the phosphoinositides. Similar activation of Heterotrimeric G protein during folic acid chemotaxis has been reported, however, downstream signaling events have not been investigated and are not well understood. We have developed a robust chemotaxis protocol which allows us to use folic acid as a chemoattractant to investigate cellular responses. Even though Ras is implicated in cAMP chemotaxis, Ras C, Ras G and Ras CG null cells were found to be capable of chemotaxing towards folic acid. We are now investigating the activation of Ras, the localization of PI3K-GFP and the synthesis of PIP3 in wild-type and mutant cell lines upon uniform folic acid stimulation and during folic acid chemotaxis. These data suggest that similar downstream signaling and cytoskeletal proteins are activated in cAMP and folic acid-mediated chemotaxis.

301/B248
**Hax1 Regulation of Neutrophil Chemotaxis.**
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Severe congenital neutropenia (SCN) is a heterogeneous group of primary immunodeficiency disorders characterized by neutropenia and life threatening bacterial infections. SCN include inherited disorders caused by homozygous mutations in HS1 associated protein X-1 (HAX1).
Hax1 is an adapter protein that interacts with the Arp2/3 binding protein and hematopoietic cortactin homologue HS1. We have generated Hax1-deficient PLB-985 neutrophil-like cells using lentiviral shRNA. Here we show that Hax1-deficient PLB-985 neutrophil-like cells have impaired directed cell migration and demonstrate an elongated morphology consistent with a retraction defect. Using microfluidic chemotaxis chambers, we show that Hax1-deficient cells show reduced migration speeds in gradients of chemoattractants. Endogenous Hax1 showed localization to the uropod during random motility and targeted to the leading edge during directed migration. Preliminary data suggest that Hax1 depletion may affect the activity of Rho GTPases. These findings suggest that Hax1 mutations may contribute to congenital neutropenia by affecting neutrophil motility and trafficking.

302/B249
**Gβγ Signaling Regulates Primordial Germ Cell Migration in Zebrafish.**
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Primordial germ cell (PGC) migration in zebrafish serves as an excellent model for the study of long-distance cell migration within a complex and developing intact organism. It has been established that the chemokine SDF1a and its cognate G protein-coupled receptors, Cxcr4b and Cxcr7, constitute a signaling axis that is essential for proper PGC migration. However, how G proteins and their downstream effectors contribute to this process remains poorly understood. Here we show that signaling mediated by the G protein βγ subunits is required for the directionality of PGC migration in zebrafish. Embryos with PGC-specific overexpression of a Gβγ scavenger, Gαt or a dominant-negative form of Gγ2 that disrupts Gβγ function, exhibited ectopic PGC localization. Analysis of cell migration behaviors revealed that PGCs in embryos with compromised Gβγ function were unable to migrate directionally toward a chemoattractant, and that they migrated at reduced speed. Furthermore, confocal time-lapse imaging showed that compared to WT PGCs, which are polarized in the direction of migration and migrate actively to a source of chemoattractant, PGCs with disrupted Gβγ signaling failed to become polarized and extended “bleb”-like protrusions in multiple directions, which caused them to migrate in random directions. Finally, in the chemo-attractant experiment PGCs with disrupted Gβγ function failed to respond to the exogenous chemoattractant Sdf1. Collectively, our findings support a crucial role for Gβγ signaling in Sdf1a/Cxcr4b-regulated PGC migration. We are currently investigating the molecular mechanisms by which Gβγ signaling regulates PGC migration. Given that the signaling pathways involved in cell migration are highly conserved during embryogenesis, the immune response and cancer-cell metastasis, our studies in an animal model with a vertebrate body plan are likely to provide new insights into the regulation of cell movement in humans, both during normal development and in the context of disease.

303/B250
**Cryo-Electron Microscopy and Single-Particle Analysis of MSP Filaments Derived from the Amoeboid Sperm of Ascaris suum.**
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Locomotion of nematode sperm is remarkably similar to that of most other crawling cells but is powered by a system of filaments composed of major sperm protein (MSP) instead of the actin-myosin machinery typically associated with amoeboid motility. The MSP motility apparatus has been reconstituted in vitro, and individual MSP filaments can be generated by either of two methods: the addition of ethanol or other water-miscible alcohols to purified MSP (King et al. 1992. JCS 101:847) or by the addition of ATP to detergent-treated Ascaris sperm cytosol. Filaments obtained by both methods have been successfully vitrified and examined by cryo-electron microscopy, thereby enabling detailed structural comparison of filaments formed from purified protein to those assembled in the presence of the other components of the motility apparatus. Both preparation techniques utilize steps that remove unwanted components, namely
excess ethanol or other cytosolic proteins, which hinder vitrification and obscure filament structure. We sampled individual filaments along their lengths and generated three-dimensional reconstructions by single-particle analysis using the Spider software suite. Comparing the cryo-EM models, as well as comparisons with MSP filament models derived from helical diffraction of negative-stained filaments formed in ethanol or by x-ray crystallography (Bullock et al. 1998. NSB 5:184) suggest important differences between filaments formed under physiological and nonphysiological conditions. There are currently six *Ascaris* sperm proteins known to modulate MSP filament dynamics in sperm; these same proteins are absent from filaments prepared using purified MSP. Comparing MSP filament models with and without these accessory proteins allows us to map the binding sites of these accessory proteins and provides a greater understanding of how they effect MSP filament dynamics and influence motility. Supported by NIH Grant R37 GM29994 and by the American Heart Assoc.

**304/B251**

**The Effect of Monothioglycerol in the Freezing Extender for Boar Sperm Cryopreservation.**


Cryopreservation and In Vitro fertilization (IVF) protocols are important in genetic studies and applications to transgenic animals. Various studies about boar sperm cryopreservation have been studied for a long time. Those were about the use of extenders, the choice of sugars, the cooling and warming rates. Furthermore our study focused on the integrity of genetic materials. The factors that influence the boar sperm are the dramatic changes in temperatures, osmotic and toxic stresses, and reactive oxygen species (ROS) generation. Among these factors, ROS generation is the main damage to DNA which is a principal genetic material and the most important for the practical applications. So we wondered whether ROS generation could be reduced. In previous study, monothioglycerol (MTG) was essential for the culture of embryo stem cells. Therefore we added MTG in the freezing extender based on lactose-egg yolk (LEY) with trehalose. For the assessment of the frozen-thawed sperm, we focused on DNA damage. First, we used a computer-aided sperm analysis system for overall conditions of sperm such as motility and viability. Then we performed the sperm chromatin structure assay for DNA integrity. And our result showed the existence of MTG in the freezing extender caused less damage to DNA in frozen-thawed boar sperm. We concluded that this reagent is due to the oxidation of lipids and proteins that participate in the fusion and subsequent penetration of the oocyte by sperm and the characteristic of MTG which is reactive with oxidizing agents would be helpful because ROS could reduce ROS generation which causes less damage to DNA in sperm.

**305/B252**

**Evaluation of Antifreeze Protein (AFP) on Boar Sperm during Cryopreservation.**

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Research for optimal cryoprotectant of cryopreservation is very important and has been studied in the field of storing sperm in freezing at low-temperature. In these days, glycerol has been used for many studies. But, glycerol has cytotoxicity that induces changes in the lipid packing structure of the cell membrane. Otherwise, antifreeze proteins (AFPs) had been reported to protect cells in low temperature from cold shock damage. So, we applied AFPs to boar sperm cryopreservation and estimated AFPs as the optimal cryoprotectant to boar sperm in cryopreservation. The boar semen were centrifuged for 3 minutes at 2,400 g, 15 degree of Celsius and then separated to sperm from the semen by Hulsen solution. The sperms were diluted with the BF5 solution. BF5 diluent, an extender, mixed with glycerol and AFPs. Control group was the AFPs-free extender. Sperm was freezed by programmable freezer and stored in liquid N2. We measured sperm viability and intact acrosome status as parameters to evaluate the condition of boar sperm added AFP. To determine the status of the viable population of cells, a double staining technique (SYTO16 and PI) was used in fluorescence activated cell sorting (FACS). Also, intact acrosome
status was tested by using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA). We observed sperm, stained FITC-PNA, using fluorescence microscopy. In these experiments, Sperm quality was enhanced by extender with glycerol containing AFPs, in comparison with that with glycerol only. It suggests that AFP have effect of increasing efficiency for sperm cryopreservation. This research fund was performed KOPRIS PE09070.

306/B253  
**Light Effects on Cell Adhesion and Motility in Diatoms.**  
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Diatoms are single celled algae that move via musculagel-mediated contact and translocation with solid underling substrates. As such, diatom adhesion is directly associated with motile activity. Abiotic conditions that influence diatom adhesion and motility thereby have dramatic consequences for cells in environments such as streams where cell colonization requires proper attachment and movement onto available surface areas in the presence of local water flow. In order to test the effects of light on diatom adhesion in natural water flow settings, our lab has tested the ability of diatoms to remain adhered to solid glass or tumbled marble substrates in an artificial flume in which both light and water flow could be controlled. Using a water speed of about 10 cm/sec, cells were placed onto substrates and allowed to adhere in the presence of either dim (ca. 50 umol/m2/s) or bright (ca. 250 umol/m2/s) light, then measured for percentage of cell retention over 3 minutes. Ability of cells to be retained showed a strong relationship to both light exposure and type of substrate. on the marble substrate, *Pinnularia* cells exposed to bright light lost much of their ability to stay adhered (7.4 ± 0.9% remaining adhered compared to 37.6 ± 7.6% remaining adhered under dim light; P =.02), but showed no light-dependent loss of adhesion when incubated on the glass surface. In contrast, *Stauroneis* cells showed a weakly significant (P=.05) greater adhesion to glass (7.8 ± 1.3% remaining adhered on glass compared to 4.5 ± 0.7% on marble) that did not show significant light dependence, although the adhesion in the presence of blue light generally did result in more cells adhering than in either bright red light or dim natural light. By comparing the flume results with those from tests on cell motility responsiveness (which show *Pinnularia* more sensitive in the blue wavelengths and *Stauroneis* more sensitive in the blue and red) we can better determine the environemental conditions under which different diatoms can move and colonize onto surfaces most effectively. This work was supported by DePaul University Research Council, the DePaul College of Liberal Arts & Sciences, and equipment purchased through NSF Grant IBN-9982897.

307/B254  
**Zyxin Regulates Delamination of Migratory Cells during Embryonic Chick Tri-Geminal Placode Morphogenesis.**  
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Self assembly of tissues and organs during embryogenesis is dependent on dynamic regulation of cell shape and cell-cell adhesion. Epithelial cells adhere through cadherin-based junctions, which are coupled to the actin cytoskeleton by actin regulatory and binding proteins. Epithelial cells retain the ability to revert to a non-adherent state, dismantling adherens junctions and becoming migratory, in the process of epithelial-mesenchymal transition (EMT). Live cell imaging of cultured MDCK cells expressing zyxin mutants, induced to undergo EMT by HGF treatment, demonstrate that the actin regulatory protein, zyxin, regulates EMT response by mediating linkage between dynamic contractile actin networks and the cell-cell junctional membrane. Constitutively active zyxin mutant expression results in a scattering phenotype in which cells become migratory but fail to fully disrupt cell-cell adhesions, resulting in a partial EMT response in which cells migrate while remaining cell-cell adherent. These effects suggest that zyxin may regulate the ability of migratory epithelial cells to detach during morphogenegetic rearrangements of embryonic development. To verify this idea, we characterize effects of mutant zyxin expression on trigeminal placode development in an embryonic chick model. During trigeminal placode development individual and small groups of cells delamate from the ectoderm, invade the
mesenchyme and coalesce to form the placode. Zyxin mutants were introduced into trigeminal placode precursor cells by electroporation and placode cells’ position during subsequent development monitored. Supporting our hypothesis that zyxin regulates coordination of migratory behavior between cells in cell-cell contact, we observe that electroporation with constitutively active zyxin results in trigeminal placode precursor cells remaining in the ectoderm, rather than invading into the mesoderm as observed in non-electroporated controls. A putative dominant negative zyxin mutant has no effect on placode cells’ delamination and invasion. Zyxin effects on placode morphogenesis recapitulate observed effects in cell culture, confirming zyxin’s role in the coordination of morphogenetic cell movements during embryogenesis.

**308/B255**

**GRASP and IPCEF Promote ARF to Rac Signaling and Cell Migration by Coordinating the Association of ARNO/Cytohesin 2 with Dock180.**

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ARFs are small GTPases that regulate vesicular trafficking, cell shape and movement. ARFs are subject to extensive regulation by a large number of accessory proteins. The many different accessory proteins are likely specialized to regulate ARF signaling during particular processes. ARNO/cytohesin 2 is an ARF-activating protein that promotes cell migration and cell shape changes. We report here that protein-protein interactions mediated by the coiled-coil domain of ARNO are required for ARNO induced motility. ARNO lacking the coiled-coil domain does not promote migration and does not induce ARF-dependent Rac activation. We find that the coiled-coil domain promotes the assembly of a multiprotein complex containing both ARNO and the Rac-activating protein Dock180. Knockdown of either GRASP/Tamalin or IPCEF, two proteins known to bind to the coiled-coil of ARNO, prevents the association of ARNO and Dock180 and prevents ARNO-induced Rac activation. These data suggest that scaffold proteins can regulate ARF dependent processes by biasing ARF signaling toward particular outputs.

**309/B256**

**Atypical PKC Zeta Regulates Recruitment, Release and Activation of MMP-9 at Podosomes.**

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Podosomes are transient cell surface structures essential for degradation of extracellular matrix during cell migration and invasion. Protein kinase C (PKC) is involved in the regulation of podosome formation; however the roles of PKC isoforms in podosome formation and proteolytic function are largely unknown. Recently, we reported that phorbol-12, 13-dibutyrate (PDBu), a PKC activator, induced the formation of podosome-like structures in primary normal (non-cancerous) human bronchial epithelial cells. Here, we demonstrate that PDBu-induced podosome formation is mainly mediated through translocation of conventional PKCs, especially PKCα, from the cytosol to the podosomal plasma membranes. Interestingly, although blocking atypical PKCs did not affect podosome formation, it significantly reduced matrix degradation. Inhibition of atypical PKCζ reduced PDBu-induced matrix degradation at podosomes through less recruitment of MMP-9 to podosomes, and decreased local MMP-9 release and activation. Down-regulation of MMP-9 by siRNA and neutralization antibody also significantly reduced matrix degradation. PDBu-induced recruitment of PKCζ and MMP-9 to podosomes was blocked by novel PKCs inhibitor Rottlerin and PKCδ siRNA. In summary, our data suggest that although the redistribution of classical PKCα controls podosome formation and dynamics, it is the atypical PKCζ (which is recruited by novel PKCδ to podosomes) that locally control the proteolytic activity of podosomes.
310/B257
Cdk5 Limits Src Activity by Targeting C-Src for Ubiquitin-Dependent Degradation.
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Purpose: Activity of the non-receptor tyrosine kinase, cSrc, is stringently controlled by inhibitory phosphorylation of a C-terminal tyrosine and by Cullin5-dependent ubiquitylation and degradation of the active form, Src(pY419). Recent studies from this laboratory have shown that inhibition of Cdk5 in spreading cells increases cSrc activity and prevents stress fiber contraction in a Src-dependent manner, demonstrating that Cdk5-dependent regulation of Src activity is physiologically relevant. The present study was undertaken to explore the mechanism underlying regulation of Src activity by Cdk5. Methods: Human lens epithelial cells (FHL124) were transfected with cDNA for the indicated fusion proteins. V5-tagged proteins were isolated by immunoprecipitation and immunoblotted for Src(pY419), V5, and ubiquitin. Src activity was measured as the ratio of Src(pY419)/total Src. Cdk5 activity was blocked by 15 microM olomoucine, Cdk5(D144N) (dominant negative), or siRNA oligonucleotides. Proteosomal degradation was inhibited with 50 microM Z-L3VS. Results: Inhibiting Cdk5 activity by three independent methods increased Src activity 50-70%. Suppressing Cullin5 expression increased Src activity 2 +/- 0.2 fold, and abolished the effect of Cdk5 inhibition. V5-tagged cSrc(S75A), which lacks a potential Cdk5 phosphorylation site, was 2-2.5 fold more active than the wild type protein when expressed in cells. Following inhibition of proteosomal degradation, incorporation of ubiquitin into Src(S75A) was approximately 77% of wild type Src. Since ubiquitylation rate is proportional to the concentration of active Src(pY419), the calculated rate of Src(S75A) ubiquitylation is approximately 40% that of wild type Src. Conclusions: Cdk5 limits Src activity by increasing the rate of ubiquitylation by Cullin5. This effect requires an intact phosphorylation site at Src(S75), a site known to be phosphorylated by Cdk5 in vitro.

Dynein (311 - 333)

311/B258
Morphogenesis of the Endoplasmic Reticulum (ER) and the ER-Golgi Intermediate Compartment (ERGIC): Requirement for Dynein-Dynactin, Lis1, and Spectrin, but Not for Membrane Proteins.
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Models for the generation of membrane tube networks, such as those of the ER, implicate the morphogenic membrane proteins of the reticulon/Yop1p families. Yet, these models do not account for the participation of the microtubule cytoskeleton in the generation and maintenance of ER in vivo. Here, we report that, in neurons, the cytoplasmic dynein machinery, Lis1, spectrin, and the microtubules are essential for the structural integrity of the ER and ERGIC. We found that βIII spectrin and reticulon 4 (ret4), a resident protein of the reticular ER, localize to microtubule-tethered filamentous/vesicular structures; cold-induced depolymerization of microtubules leads to fragmentation and peripheral dispersion of ret4- and βIII spectrin-labeled structures, a response typical for the loss of activity of cytoplasmic dynein. Downregulation of βIII spectrin, a protein that recruits dynein-dynactin to membranes, or of Lis1, a regulator of cytoplasmic dynein activity, disperses the ret4 network and the ERGIC53-positive tubulo-vesicular structures. Thus, the maintenance of the reticular ER and ERGIC relies on their interaction with the microtubules. These results could be explained by the ability of dynein-dynactin to bind to acidic phospholipids within membranes via spectrin, and extension of the anchored tubes by moving along microtubules. In support of this hypothesis, with purified proteins, we show that a mixture of cytoplasmic dynein, microtubules, and liposomes containing acidic phospholipids generates elaborate networks of interconnected membrane tubes (with ER-characteristic ring closures and
three-way junctions), and freely moving tubulo-vesicular clusters, typical for the ERGIC. With this reconstituted system, we further show that the formation of tubes requires dynactin and spectrin, and is regulated by phospholipase D and small GTPases. Taken together, these results indicate that similar mechanisms underlie the generation and maintenance of ER-like tube networks and small tubular transport entities, by the action of soluble microtubule motors and anchoring proteins, which bind to phospholipids, not to membrane proteins. Funded by March of Dimes (1-FY04-240) and NIH (R01GM068596).

312/B259
The Role of Dynactin in Dynein-Based Intracellular Transport.
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A diverse number of cellular functions require the cytoplasmic dynein and dynactin motor complex to generate force for movement. Cytoplasmic dynein is essential for organelle distribution and transport, mitotic functions, microtubule and neurofilament transport, and retrograde axonal transport. Long-range movements of retrograde cargo, ranging from microns to meters, are particularly important in the axonal processes of neurons. Dynein processivity, the ability to take multiple steps without dissociating from the microtubule, is enhanced by dynactin. Mutations in the cytoplasmic dynein and dynactin motor complex could result in severe neuronal disorders by altering axonal transport. The aberrant function of the motor complex has been implicated in a vast number of human diseases, suggesting that dynactin may be a useful target for treatment of neurodegenerative diseases and motor neuron diseases, such as amyotrophic lateral sclerosis, or ALS. We are employing several intracellular trafficking assays to examine the role of dynactin in dynein-based motility. Utilizing GFP-labeled cargoes and BODIPY-ceramide, we are analyzing the effects of alterations in dynactin on the motility of cargoes inside a variety of mammalian tissue culture cell lines.

313/B260
Direct Observation of Dynein/Dynactin Complex Dynamics in MAP2c Induced Microtubule Bundle Motility.
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In neuronal and non-neuronal cells, MAP2c decorated microtubule bundles are under the influence of a dynein-dependent pushing force. If such microtubule bundles are free to move, they are rapidly transported through the cell. at the cell periphery, such bundles can exert an outward force that is opposed to an actin mediated inward force. Changes in the balance of these forces can lead to the induction of new neurite-like cell protrusions. Here, we directly observed cortical dynein/dynactin complexes and motile microtubule bundles via wide-field and TIRF microscopy. Cortical dynein/dynactin complexes are preferentially associated with microtubule bundles that come in close proximity with the plasma membrane in the TIRF field. We find that at least two different dynein/dynactin populations exist, that are either stationary or associated with the minus ends of motile microtubule bundles. Our results support a model in which free dynein complexes are transported towards the microtubule bundle minus-end, while stationary, cortex associated complexes can push microtubules directionally with leading plus-ends, thereby focusing a dynein-mediated force to locally induce neurite-like cell protrusions.

314/B261 ABSTRACT WITHDRAWN

315/B262
Madmax-1 Organizes Dynein-Dependent Secretory Transport in Developing Neurons.
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Membrane and secretory trafficking are essential for proper neuronal development. However, the molecular mechanisms that organize secretory trafficking are poorly understood. Here, we identify Madmax-1 as an effector of the small GTPase Rab6 and key component of the molecular machinery that coordinates dynein-dependent secretory vesicle transport. We show that Madmax-1 has homology to Bicaudal-D (BICD) family proteins, interacts with the dynein/dynactin retrograde motor complex, organizes Rab6-positive secretory vesicle exocytosis and is required for neural development in zebrafish. In developing neurons, Madmax-1 expression strongly declines during early neurite outgrowth. Sustained expression of Madmax-1, as well as knockdown of Rab6 suppresses neuritogenesis. In contrast, sustained expression of BICD2 does not significantly affect neurite development. These results indicate an important developmental role for Madmax-1 as an organizer for dynein-dependent trafficking of Rab6-decorated secretory vesicles. The data uncover a new mechanism to achieve regulation and specificity in secretory trafficking and exocytosis during neuronal development.

316/B263
Motor Coordination via Tug-Of-War Mechanism Drives Bidirectional Vesicle Transport.
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The microtubule motors kinesin and dynein function collectively to achieve long-range, bidirectional transport in the cell. While previous work suggests kinesin and dynein motors may be bound to cargos simultaneously, the mechanisms that regulate bidirectional motility are not well understood. Analysis of axonal transport in live cells reveals that multiple modes of transport may be observed concurrently. Cargos exhibit fast, unidirectional movement in both anterograde and retrograde directions, as well as saltatory, bidirectional movement and pausing. To characterize bidirectional transport in a simplified In Vitro system, we purified endogenous cargos from transgenic mice with neuronally expressed GFP-dynamitin. We isolated fluorescent vesicles ~90 nm in diameter that are enriched for markers of the axonal transport compartment. These purified axonal transport vesicles bind to and translocate along microtubules in an ATP-dependent manner. Quantitative photobleaching and western blot analysis indicate that 3-6 dynein motors and one kinesin-1 motor were stably bound to each vesicle. In Vitro vesicle motility is bidirectional, similar to the saltatory motility seen in cellular assays. High-resolution analysis using total internal reflection microscopy indicates that directional switches are correlated with off-axis movement, and are likely due to stochastic switching between vesicle-bound kinesin and dynein motors. Inhibitory antibodies to dynein modulate the net direction of transport, consistent with this hypothesis. We compared the predictions of a simple tug-of-war model to the observed trajectories, velocities, and directional motility of vesicles in vitro, and good agreement was found assuming 6 dynein motors and 1 kinesin motor are active. The inhibitory effects of antibodies were modeled as a reduced number of active dynein motors, and the simulated trajectories compare well with the observed experimental trajectories. Together, our observations and modeling results indicate that vesicles move robustly with a small complement of motors, and suggest an efficient regulatory scheme where small changes in the number of engaged motors manifest in large changes in the motility of the cargo.

317/B264
Dynein Drives Autophagosome Motility in HeLa Cells.
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Dynein, an essential microtubule-based motor for retrograde axonal transport, plays a central role in autophagy, a process by which defective cellular components are degraded by the lysosomal machinery. Dynein has been proposed to drive the transport of autophagosomes to the cell center, allowing for effective fusion with lysosomes and clearance of toxic cellular material (Köchl et al., 2006; Jahreiss et al., 2008; Kimura et al., 2008). Disruption of either dynein function or autophagy is sufficient to cause neurodegeneration marked by the accumulation of protein aggregates. We aim to understand the mechanisms that regulate autophagosome transport and how this process is misregulated in neurodegenerative diseases. To develop an assay to monitor autophagosome motility and localization, we transfected HeLa cells with GFP-LC3, a marker for autophagosomes. We found that treatment of HeLa cells with either nocodazole or vinblastine to depolymerize microtubules results in an accumulation of autophagosomes, as evidenced by an increase in GFP-LC3 puncta. Microtubule depolymerization also disrupted the predominant perinuclear localization of LC3, which became distributed throughout the cytoplasm. To examine dynein function in autophagosome localization, we overexpressed the coiled-coil region (CC1) of the p150Glued subunit of dynactin, an activator of dynein. CC1 blocks dynein-dynactin interactions and disrupts dynein-based motility. Overexpression of CC1 in HeLa cells dispersed autophagosomes to the cell periphery. This effect was enhanced by starvation in Earle's balanced salt solution in the presence of bafilomycin A1, an inhibitor of the vacuolar H+-ATPase. Live cell imaging of HeLa cells revealed much bidirectional movement of LC3 and retrograde transport events towards the perinuclear region were observed. These results, together with previous observations, indicate that dynein drives the transport of autophagosomes along the microtubule network in HeLa cells. These observations will serve as a foundation to dissect this process at a mechanistic level and to study how defects in this pathway may contribute to neurodegeneration by failing to eliminate toxic protein aggregates.

318/B265
Mechanical Cues Induce Asymmetric Dynein Distributions Driving Large-Scale Movements in the Cell.

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Motor proteins, exerting force on microtubules, position nuclei, spindles, and other organelles in eukaryotic cells. Hence a central question is how a multitude of motor proteins and microtubules work together to produce large-scale movements of organelles in the cell. The vigorous nuclear oscillations during the meiotic prophase of the fission yeast Schizosaccharomyces pombe provide an excellent system to study organelle movements dependent on cytoplasmic dynein and microtubules. These oscillations span the whole cell and are crucial for proper chromosome pairing and recombination, yet the mechanism of the oscillations was unknown. We provide a mechanism of these oscillations based on collective behavior of dynein motors linking the cell cortex and dynamic microtubules that extend from the spindle pole body in opposite directions. By combining live cell imaging, laser ablation and quantifying the number of dynein motors with a theoretical description, we show that dynein dynamically redistributes in the cell in response to load forces, resulting in more dynein attached to the leading than to the trailing microtubules. The redistribution of motors introduces an asymmetry of motor forces pulling in opposite directions, leading to the generation of oscillations. Our work provides the first direct In Vivo observation of self-organized dynamic dynein distributions, which, due to the intrinsic motor properties, generate regular large-scale movements in the cell. We propose a novel mechanism for spatio-temporal pattern formation of motor proteins governed by mechanical cues that differs from conventional molecular signaling, as well as from self-organization based on a combination of biochemical reactions and diffusion.

319/B266
LIS1 and Nude Allow Multiple Dynein Motors to Cooperate to Transport High Loads.

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Cytoplasmic dynein is involved in a wide range of intracellular movements including fast vesicular transport and slow nuclear translocation. How one motor contributes to fast, low load movement as well as slow, high-load movement is unknown. We have found that two dynein regulatory factors, LIS1 and NudE, cooperate to convert dynein to a novel persistent force state under load (Mol Biol Cell 19 (suppl.), 1546). We found that NudE recruits LIS1 to dynein to form a triple complex, in which LIS1 binds the dynein motor domain in an ADP-VO4 transition state-specific manner. LIS1 enhanced the affinity of dynein for microtubules, but only under transition state conditions. In single molecule bead assays, LIS1 dramatically prolonged the interaction of dynein with microtubules under load, providing the first evidence for a role in dynein force regulation. We have since examined the effects of LIS1 and NudE on the dynein-microtubule interaction in further detail. To measure the resistance of dynein to detachment from microtubules directly, we used “superforce” analysis. Beads bearing single dynein molecules were subjected to sudden increases in laser trap strength to exceed the dynein stall force, and the time to detachment from microtubules was determined at high precision. LIS1 alone or in combination with NudE extended the detachment time by up to 5-fold. To test how LIS1 and NudE affect dynein force production under multi-motor conditions, we incubated beads with dynein at high concentration and monitored force production at 3.7 pN trap strength. Beads coated with ~2-3 dynein molecules showed an increased frequency of multi-motor events with addition of LIS1 and NudE, which also caused a dramatic increase in trap escape. In silico simulations confirm that the prolongation of individual dynein-microtubule interactions should result in enhanced force production by multiple motors. These results appear to explain the need for LIS1 and NudE in dynein-dependent, high-load intracellular movements, where multiple motor activity is critical. Structure-function analysis of the relative contributions of LIS1 and NudE to these aspects of dynein behavior is under way. Supp. by GM47434, HD40182, and GM070676.

**320/B267**

*Construction of BiFC Fusions to Assay for In Vivo Interactions among Dynein HC, Pac1/LIS1, and Bik1/CLIP-170 at Microtubule Plus Ends.*

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Pac1/LIS1 and Bik1/CLIP-170 are two key proteins required for the targeting of dynein to cytoplasmic microtubule plus ends in budding yeast. We have previously proposed a model whereby Pac1/LIS1 binds to the motor domain of the dynein heavy chain, Dyn1/HC, forming a complex that interacts with the +TIP protein Bik1/CLIP-170 at plus ends. Here, we utilized Bimolecular Fluorescence Complementation (BiFC) probes derived from Venus (VN, N-terminal 172 amino acids of Venus; VC, C-terminal 83 amino acids of Venus) to assay for protein-protein interactions among Pac1/LIS1, Bik1/CLIP-170 and Dyn1/HC. We observed that cells co-expressing Dyn1-VN with Pac1-VC or Bik1-VC exhibited fluorescent foci associated with microtubule plus ends, the cell cortex, and the spindle pole bodies (SPBs). Additionally, cells co-expressing Pac1-VC with Bik1-VN exhibited fluorescent foci associated with microtubule plus ends. As controls, strains expressing Dyn1/HC, Bik1/CLIP-170 or Pac1/LIS1 fused with either VN or VC exhibited no fluorescence signal above background. These data support the model that these three components associate in a complex at the plus end of microtubules.

**321/B268**

*Pathway in Dynein Targeting to a Microtubule plus End: Differential Roles of Pac1/LIS1 and Bik1/CLIP170.*

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In S. cerevisiae, targeting of dynein and dynactin complexes to the microtubule plus end is a pivotal step of the dynein pathway, which is required for the movement of the mitotic spindle into the bud neck during anaphase. A total of thirteen motor and regulatory components of the dynein pathway are targeted to the plus end. Knowing the copy number of each of these components at one microtubule plus end is necessary to understand the molecular architecture of the plus-end complex, and to elucidate the mechanisms underlying dynein function. We have counted, with molecular accuracy, the number of each component using quantitative fluorescence microscopy. Our measurements reveal novel subunit stoichiometry for the dynein and dynactin complexes. Additionally, using bimolecular fluorescence complementation (BiFC), we have demonstrated In Vivo interactions between dynein, Pac1/LIS1 and Bik1/CLIP170, and mapped an interaction pathway for dynein plus-end targeting. We found that a stoichiometric amount of Pac1/LIS1 and an excess of Bik1/CLIP170 link the dynein complex to the microtubule plus end via the motor domain of Dyn1/HC. Pac1/LIS1 and Dyn1/HC are dependent on each other and on Bik1/CLIP170 for plus-end targeting, whereas Bik1/CLIP170 is independent of either. Furthermore, overexpression of Pac1/LIS1 enhances Dyn1/HC targeting, and vice versa. These data suggest that dynein and Pac1/LIS1 assemble as a 1:1 pre-complex that then associates with Bik1/CLIP170, which we propose mediates direct interaction with the plus end. Our findings allow us to visualize the overall arrangement of a functional plus-end complex at a single microtubule plus end.

322/B269

Interactions of the I1 Intermediate Chain Dyneins IC140 and IC138.
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The dynein microtubule motors belong to class of ubiquitous and essential family of molecular motors that are involved in many vital biological processes such as left-right patterning during development, chromosome segregation, vesicular trafficking, and ciliary and flagellar motility. This work focuses on the role of axonemal dynein intermediate chains in targeting the I1 dynein complex to the microtubule doublet using *Chlamydomonas reinhardtii* as a model system. The I1 dynein complex is targeted and anchored to a unique position on the A-microtubule of the doublet yet the mechanism by which this happens has yet to be fully elucidated. Chemical cross-linking studies revealed that the inner arm dynein I1 intermediate chains IC138 and IC140 each independently form complexes of approximately 200 kDa and 300 kDa when treated with a zero-length cross-linker. We have determined that the smaller cross-linked product is a complex containing the I1 intermediate chain (IC140 or IC138) and tubulin. Furthermore, 200 kDa cross-linked complex is capable of forming in the presence of purified I1 (f-dynein) and taxol-stabilized microtubules only. These results indicate that not only can the dynein intermediate chains bind directly to the microtubule but no other non-I1 proteins are required to mediate that interaction.

323/B270

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Dynactin is a multi-subunit complex that links microtubule-based motors to the cargo they transport. Loss of some subunits (Arp1, Arp11 and p62) leads to loss of the entire dynactin complex and results in aberrant mitotic spindle formation and endomembrane mislocalization. However, the core dynactin structure is unperturbed in the absence of the p25 and p27 subunits, indicating that these proteins are not required for dynactin stability. Cross-linking of native dynactin reveals that p25 and p27 are closely associated with each other. These proteins are distal components of the “pointed end complex” which is involved in cargo specification. In addition to the dynactin-associated pool, cells contain free p25/p27 heterodimers that are not associated with other dynactin subunits. p25 and p27 are genetically related and are predicted to adopt a left-handed beta-helical fold. We have mapped the epitope recognized by the p27 monoclonal antibody, mAb 27A, to an alpha-helical loop that projects away from the beta helix. In fixed cells and cytosols, mAb 27A recognizes the free pool of p25/p27 heterodimers but not the dynactin-associated pool, suggesting that this surface loop is masked in native dynactin, most
likely by other subunits. We are undertaking cross-linking analysis of purified recombinant p25/p27 heterodimers in order to reveal the binding interface between these two proteins. This analysis may also provide information about the solvent-exposed surface. This work should provide important new information regarding the way in which p25 and p27 bind each other and will also provide new insights regarding possible mechanisms for dynactin and cargo-binding.

324/B271
The Dynamitin N-Terminus: A Potential Link between the Two Structural Domains of Dynactin.
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Dynactin, a multi-subunit complex, works with dynein and other motors in a wide range of subcellular activities. Its subunit dynamitin (p50) is essential to its structural integrity. Over-expression of dynamitin dissociates the dynactin subunit p150glued from the Arp1 minifilament in vivo and in vitro. This suggests that dynamitin links the dynactin shoulder-sidearm domain (p150, dynamitin and p24) to the Arp1 filament. The extreme N-terminus of dynamitin N-DM (AA 1-90) is sufficient to disrupt dynactin in vivo and in vitro, yielding the same phenotypes as full-length dynamitin. N-DM's ability to disrupt dynactin in cells is unexpected, since a slightly smaller recombinant fragment (AA 1-81) of the dynamitin N-terminus does not disrupt or bind stably to purified bovine dynactin. To identify proteins that can interact with N-DM, we performed tandem affinity purification and found that N-DM pulls down the subunits of the Arp1 filament (consisting of Arp1, CapZ, Arp11, p62, p27, p25 and actin) but not the shoulder-sidearm subunits. Similar results were obtained by immunoprecipitation of myc-tagged N-DM. An in vitro binding assay using purified dynactin has further shown that N-DM can bind the Arp1 filament directly, and that other cytosolic factors are not required. Analysis of lysates lacking the dynactin p27/p25 subunits reveals that these subunits are not required for N-DM binding to the Arp1 filament. CapZ does not bind N-DM in lysates lacking Arp1 and Arp11, suggesting that N-DM binds Arp1 directly. Over-expression of N-terminal fragments of dynamitin enhances the formation of Arp1 cables, suggesting that the dynamitin N-terminus stabilizes Arp1 polymers. Together, our findings suggest an interesting new model of dynactin structure.

325/B272
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Besides its physiological binding partners, cytoplasmic dynein can be recruited by pathogenic cargo, such as viruses for intracellular motility. Incoming adenovirus 5 (Ad5) is known to use dynein-mediated transport toward the nucleus after its escape from the endosome. Our lab has previously reported that this interaction involves the adenovirus capsid protein hexon (MBC, 2007, 18(suppl):1661). Biochemical, immunolocalization, and phenotypic analysis also indicated that the interaction with cytoplasmic dynein is direct and appears not to require the known adaptors ZW10, NudE, or dynactin. We now report that short-term exposure of adenovirus or purified hexon to decreasing pH strongly enhances the subsequent interaction with purified rat brain cytoplasmic dynein at neutral pH. Using pH-optimized binding conditions we find the dynein intermediate chain (IC) and light intermediate chain 1 (LIC1) expressed in Cos-7 cells interact with hexon, whereas LIC2 and the dynein light chains RP3, TcTex-1 and LC8 do not. Hexon-IC binding does not involve the N-terminal IC residues 1-125, the known binding site for the dynein regulator dynactin. These results indicate that the passage through the acidic lumen of the endosome primes the adenovirus capsid for dynein binding. Furthermore, the capsid interacts with multiple cytoplasmic dynein subunits and - in the case of IC binding - presumably at a novel site. This is consistent with our more general observation that adenovirus recruits cytoplasmic dynein directly through a mechanism that differs from that used by physiological forms of cargo. Supp. by NIH GM47434.
326/B273
Dynein Unmasking: Spatial Regulation of Dynein Mediated by Intramolecular Associations.
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In *S. cerevisiae*, cortical dynein plays an integral role in positioning the mitotic spindle in the bud neck prior to anaphase. For dynein to associate with the cell cortex, it must be off-loaded from the plus-ends of astral microtubules emanating from the spindle pole body (SPB). While the mechanism preventing dynein from bypassing the off-loading process is unclear, we recently proposed a model whereby the N-terminal cortical association ‘tail’ domain of the heavy chain is masked by the C-terminal ‘motor’ domain until its association with plus-ends, thus precluding its ability to associate with the cortical receptor, Num1. To test whether the tail and motor domains are capable of associating *in vivo*, we attached bimolecular fluorescence complementation (BiFC) probes to the extreme termini of the dynein heavy chain (Dyn1/HC) and observed fluorescent foci associated with the SPB but not with plus-ends or the cell cortex, suggesting that this intramolecular association is spatially restricted. Into the junction between the tail and motor domains, we systematically inserted variable linker sequences to constitutively unmask Dyn1/HC. All mutants tested exhibited properties expected of a constitutively unmasked dynein motor; namely, an unregulated ability to associate with the cell cortex. This association depends on Num1, but occurs independently of Pac1/LIS1 and Bik1/CLIP-170, two proteins required for the cortical targeting of wild-type Dyn1/HC. Strikingly, when imaged using time-lapse fluorescence microscopy, we directly observed the unmasked dynein motor off-load from the plus-end to the cell cortex, lending support to the notion that unmasking is indeed a critical step in the process of delivery of cortical dynein. Taken together, our data support the notion that Dyn1/HC likely adopts a masked conformation at the SPB, is unmasked upon associating with plus-ends, which allows for the association of dynein with the cell cortex from where it mediates spindle orientation.

327/B274
The Dynactin Subunits p27 and p25 Are Membrane-Targeting Factors.
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Dynactin is well known for its roles as a cargo adaptor and processivity enhancer for cytoplasmic dynein and kinesin-2. Although dynactin is essential for mitosis and endomembrane trafficking, it remains unclear how dynactin is targeted to its various cargoes. We have used RNAi to identify the dynactin subunits p27 and p25 as membrane targeting factors. p27 knockdown selectively depletes the p27/p25 heterodimer while leaving the rest of the dynactin molecule intact, unlike Arp11 or p150Glued knockdown which compromise dynactin complex integrity and/or motor binding. Cells lacking p27/p25 form normal spindles, in marked contrast to what is seen when other subunits are knocked down. However, we find that p27 and p25 are required for centripetal movement of recycling endosomes as well as the steady state localizations of early endosomes and the trans-Golgi network. The effects of p27/p25 knockdown are specific for these particular compartments, as late endosome motility is not impaired. Membrane-associated dynactin is reduced in p27 knockdown, consistent with the hypothesis that p27/p25 is required for dynactin recruitment to specific membrane compartments. We also unravel that p27/p25 play essential roles in completion of cytokinesis, as cells lacking these subunits show defective dynactin-mediated delivery of recycling endosomes toward the midbody bridge. Our results provide new insights into the mechanism by which dynactin binds membrane cargoes and further suggest that p27/25 may serve to regulate and/or discriminate dynactin functions.

328/B275 ABSTRACT WITHDRAWN

329/B276
Processive Movement by Mutant Cytoplasmic Dynein Heterodimers with an Inactive Head.
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SUNDAY

Cytoplasmic dynein is a two-headed molecular motor, which takes hundreds steps along a microtubule (MT) without dissociation. To elucidate the mechanism of the processive motion, we expressed the heterodimeric dynein constructs, in which one head completely lost its ATP-binding activity due to the K-to-T mutation in the Walker a motif in its AAA1 module (P1T mutation). Our single-molecule motility assays showed that the heterodimeric dynein of the wild type and the P1T mutant (Wild/P1T) moved processively on MT with its velocity approximately 40% of that of the wild-type homodimer. Step size distribution for the Wild/P1T heterodimer was similar to that of the wild-type homodimer, suggesting that the Wild/P1T heterodimer also moves in hand-over-hand fashion. Because P1T mutant head in the heterodimer cannot dissociate from MT by itself, this processive motion suggests that the wild-type head pull the P1T head off from MT. Therefore, intramolecular tension between two heads is predicted to play an essential role in the processive movement of dynein. We then examined the effect of intramolecular tension through the tail domain linking the two heads. We inserted a Gly-rich flexible linker with 20 or 30 residues between the tail domain and the heterodimerizer to reduce the tension. Unexpectedly, the wild-type homodimer and the Wild/P1T heterodimer with the flexible linker moved processively; their run length and velocity were similar to those of the homo- and heterodimer without the flexible linker. These results suggest that the tension through the tail domain does not play a critical role in the processive motion. The direct interaction of the two AAA rings in the dynein heads may be responsible for sustaining alternative steps of the two heads on MT.

330/B277
Dissection of Chlamydomonas Dynein-C by Using High-Speed AFM.
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The high-speed AFM is a powerful instrument; using it, we can observe nano-meter-scale conformational change of protein molecule directly in nearly real time (~10 f/sec). By the use of the high-speed AFM in tapping mode, we have observed molecular morphology and conformational change of dynein-c molecules which were placed on mica surface. In most cases, dynein-c molecules were disrupted after several seconds’ observation, probably due to damages by the probing. However, we noticed that observation of disruption process of dynein would provide new insights in the internal structure of dyneins. In this study, we determined the linker-located side of dynein head. When we observed “left view” of dynein-c (left view: the view from perpendicular to the AAA-ring plane in which the tail protrudes to below and projecting points of the tail and stalk are left hand of the head), sometimes the dynein head was peeled off from the mica surface but the tail remained. at the same time, a hook-shaped structure appeared where the head had been placed. The hook-shaped structure was closer to the mica surface (2-3 nm) than the head surface (5-12 nm) before the peeling. The one end of the hook linked to the tail and the other end linked to the peeled head. We suppose that the hook corresponds to the linker. on the contrary, when we observed right view of dynein-c, the linker was occasionally observed through opening of disrupted head. The linker was located at almost same height to the head surface before the disruption. These indicate that the linker is located on the foreside of the head when we look at the right view of dynein-c. on the other hand, there was a ridge on the head surface of the left view. Occasionally, the majority of the ridge was peeled but one end was still linked to the head at around the position of AAA6. The peeling didn’t affect the remaining structure of the head and the tail. The tapping force of probe is so small (~10pN) that the provability of peptide-bond severing should have been very low. Taken together, we suppose that the ridge was C-terminal region of dynein-c, which would be located on the head surface when we observed the left view.

331/B278
The Role of p28C Dynein Light Chain in Ciliary Motility in Tetrahymena Thermophila.
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Axonemal dyneins drives microtubule sliding leading to ciliary motility. Cilia contain multiple dynein isoforms, The dynein light chains (LC) are thought to regulate the assembly, organization
and activity of the one headed inner arms. The work in our laboratory focuses on the p28 class of
dynein LC. A single p28 protein is present in Chlamydomonas, and it associates with a subset of
one headed inner arm dynein heavy chains (Le Dizet and Piperno, 1995). The Tetrahymena
also expresses more genes that encode one headed inner arm heavy chains than
Chlamydomonas (18 in Tetrahymena compared to 8 in Chlamydomonas) (Wilkes, 2004). These
data raise the question: Why are there three different p28 proteins in Tetrahymena? We
hypothesize that each of the light chains in Tetrahymena associate with a distinct subset of one
headed inner arm dyneins. In order to test our hypothesis, we performed qRT-PCR experiments
to determine the relative expression levels of p28. p28B was expressed at the highest
abundance, p28C at an intermediate level, and p28A at a very low level. We have initiated our
studies by introducing mutations in the p28C gene by targeted gene knockout. Southern Blot
analysis confirmed the knockout was complete. LC-MS/MS analysis of dynein HC isolated from
p28C knockouts indicated that a single inner arm dynein HC, DYH11, was missing from p28C
mutants. This suggests that p28C is required for the assembly of DYH11-containing inner arm
dynein. The observation that only one dynein HC is missing from p28C knockouts supports our
hypothesis. Behavioral analyses of the p28C mutant indicate that there is a 58% reduction in
ciliary beat frequency and 56% reduction in swimming speed compared to wild-type cells. These
data suggest that p28C associated inner arms are required for normal ciliary beat frequency in
Tetrahymena. This result was surprising since mutations in p28 affect waveform but not beat
frequency in Chlamydomonas. We are currently mutating genes p28B and a to detect whether
other p28 associated HC function in establishing waveform. Supported by NIH R15GM059855-03
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332/B279

Analysis of Dynein Heavy Chain Mutations in Neurospora crassa.
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Cytoplasmic dynein is a minus-end directed microtubule motor involved in a variety of cellular
functions. Dynein is a multi-subunit complex composed of heavy chain, intermediate chain, light
intermediate chain and light chain subunits. The heavy chain consists of an N-terminal
dimerization domain followed by six tandemly linked AAA modules that includes a microtubule
binding stalk between AAA4 and AAA5. Of the six AAA modules only AAA1-4 are thought to bind
ATP and AAA1 has been shown as the principal ATP hydrolysis site. The presence of multiple
ATP binding and hydrolysis sites adds to the complexity of dynein heavy chain structure and
poses the question of how conformational change is coordinated and regulated around the AAA
modules. In the current study we used Neurospora crassa as a model organism to study dynein
heavy chain structure-function relationships. We utilized a genetic screen to identify ropy (ro)
mutant strains that exhibit abnormal growth phenotypes that are caused by mutations in
components of the dynein/dynactin pathway. We further studied ropy (ro-1) mutant strains that
carried point mutations in the dynein heavy chain. We replaced the endogenous intermediate
chain with a fluorescently tagged intermediate chain and used this tool to localize dynein in WT
and ropy mutant strains. In the wild-type strain a bright dynein localization signal was observed at
the hyphal tips with comet-like structures in distal regions to the tip. In contrast, ropy mutant
strains exhibited altered dynein localization which includes linear tracks of dynein, loss of bright
hyphal tip localization and diffusive staining throughout hyphae.

333/B280

Proteomic and Functional Analyses of Dyneins in Cilia.
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Axonemal dyneins are molecular motors composed of heavy chains (HCs), intermediate and light
chains. HCs comprise of the motor domain and make up most of the mass of the dynein complex.
Previous studies identified eight inner arm dynein HC (DHC) in different ciliated and flagellated organisms. However, recent analyses of completed genomes of different species identified additional one-headed dynein HC genes in most of the organisms examined. An extreme example in this case is exhibited by Tetrahymena thermophila which has eighteen one-headed inner arm dynein HC genes. These findings lead to two broad questions: i) Are all predicted dyneins present in the axonemal proteome and are they all equally abundant? ii) Are all dyneins equally important for ciliary motility? To answer the first question, we analyzed axonemal DHCs in Tetrahymena thermophila by LC-MS/MS and detected fifteen one-headed DHCs. The LC-MS/MS data suggested that the HCs are present in different abundances. qRT-PCR studies also supported the fact that axonemal DHCs are expressed at different levels. These observations raise the question of whether dyneins present in different abundances play different role(s) in ciliary motility. To address that question, we knocked out two different inner arm dyneins -DYH15, the highest abundant one-headed inner arm dynein, and DYH18, present in very low abundance. Both mutants displayed a 15-25% reduction in swimming speed. Beat frequency and feeding behavior was affected in KO15, but not in KO18. Thus we can conclude that each dynein may be playing a unique role in ciliary motility. Interestingly, LC-MS/MS analyses of axonemes isolated from both the mutants revealed an increase in levels of three one-headed DHCs in KO15. No such compensation was observed in case of KO18. This raises the possibility that some dyneins are partially redundant.

Cell Migration I (334 – 364)

334/B281
Integrin-Dependent Invasive Migration in 3D Collagen of Human Lung Adenocarcinoma Cells That Survived 10Gy Irradiation.
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Radiotherapy is one of the effective therapies for various malignant tumors. However, the emergence of tolerant cells after irradiation remains problematic since the tolerant cells show high metastaticity and sometimes result in poor prognosis. Recent studies revealed that integrins play a key role in cancer cell migration and invasion. Especially, integrin-beta1 regulates cell invasion into three-dimensional collagen matrix by accumulating at the direction of cell movement. In this study, we show that tolerant cancer cells through irradiation indicate high invasive activity in 3D collagen gel and a manner of the migration is dependent on integrin-beta1. First, we established several sub-clones of human lung adenocarcinoma cells (A549 cells). Then, one of the clones named as A549P-3 was exposed to 10 Gy of X-rays. After 30 days, the surviving cells were harvested (referred to as A549P-3IR). Collagen gel overlay assay for A549P-3 and A549P-3IR was performed to observe their morphology and invasiveness. We found that most A549P-3 cells indicated round and stable morphology and low migration activity, whereas a considerable number of A549P-3IR showed spindle morphology and rapid changes in cell surface, alternating phases of elongation and retraction of protrusions, and significant increase in migration rate. Immunofluorescence staining showed integrin-beta1 accumulated at the head of elongated A549P-3IR surface but did not in A549P-3. Blocking integrin-beta1 activity using monoclonal antibody (AIIB2) inhibited A549P-3IR cell movement and altered the cell morphology into round shape. Knockdown of integrin-beta1 by RNAi also induced the same effects as AIIB2 did. These results indicate that the surviving A549 cells against irradiation acquire a sustained activity of integrin-beta1 and result in a highly invasive phenotype.

335/B282
Visualizing Rac1, RhoA, and Cdc42 Activity during Cell Migration within Three-Dimensional Extracellular Matrix.
The study of cell migration In Vitro on two-dimensional (2D) rigid surfaces has provided significant insight into the mechanisms of cell movement. Polarized intracellular signaling directs protrusion at the leading edge, adhesion to the underlying substrate, and retraction at the trailing edge to orchestrate cell motility. On 2D substrates, the Rho family GTPases Rac1, Cdc42, and RhoA are active at the leading edge, coordinating protrusion and adhesion; a second pool of active RhoA at the rear of the cell promotes actomyosin contraction to retract the trailing edge. It is important to determine whether the same mechanisms also drive cell movement within the structurally complex, three-dimensional (3D) environment found in tissues. Cell-derived extracellular matrix (ECM) provides a structurally complex 3D model system that is accessible to live cell imaging. We have now constructed and validated fluorescence resonance energy transfer (FRET)-based probes to visualize the spatial and temporal activation of Rac1, Cdc42, and RhoA within living cells. The activation-dependent binding of the intermolecular probes is detected by imaging the sensitized emission of the acceptor in the presence of the donor. Visualizing these biosensors in primary fibroblasts migrating in 3D cell-derived ECM or on glass has revealed similar intracellular localization of RhoA signaling in both environments. In contrast, Rac1 and Cdc42 activity is no longer restricted to the leading edge of cells migrating in 3D cell-derived matrix. Instead, their activity is concentrated in clusters behind leading protrusions, along the side, and at the rear of the cell. Determining how these pathways are organized in cells migrating in 3D ECM will highlight the similarities and differences in the mechanisms driving migration in 2D versus 3D environments and aid in the development of new models of cell migration.
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Recent years have seen a surge of interest in 3D systems as improved models for In Vivo cell migration. However, majority of these models are not only qualitative, they are also blind to the underlying signaling pathways that are well characterized in 2D systems. Additionally, neither the influence of matrix chemical, mechanical and structural properties nor the matrix regulation of cellular signals is understood in the context of 3D cell migration. This lack of knowledge has been a major obstacle in harnessing the full potential of 3D cell cultures. Using a combination of approaches, including bulk and microrheology, siRNA knockdown and high resolution imaging, we have answered three simple, yet critical questions in 3D cell migration. 1) How do key matrix properties such as structure, composition and mechanics independently and collectively influence matrix derived signaling during 3D cell migration? 2) How do these key matrix properties regulate cellular shape and mechanics and 3) How do these answers differ from 2D cell migration models. To answer these questions, we have quantified how the concentration of total and phospho-FAK depends on these matrix properties. We note that matrix mechanics plays a far more important role than matrix porosity or ligand density. Similarly, we have quantified that cell mechanics not only depends upon matrix structure and stiffness but also upon integrin activity. Above all, our results quantitatively suggest that cellular signaling, shape, mechanics and migration are far more sensitive to minor changes in matrix properties in 3D than they are in 2D. Small changes in mechanical properties of the matrix go virtually unnoticed in 2D but result in significant differences in migration and mechanics in 3D. Collectively, we find that cell motion as well as signals and mechanics underlying this motion depend on matrix properties in a complex and a non-linear way that is consistent with results of our mechano-chemical models.

338/B285
Cellular Control of Glial Cell Chain Migration.
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Collective cell migration is a common and fundamental phenomenon during development. The developing wing of the Drosophila provides an excellent model to investigate the mechanism of cell chain migration where a high number of cells migrate together in a head-to-tail arranged manner. Veins between the two epithelial layers of the wing contain sensory nerves and ensheathing glial cells. These latter migrate from the position where they are born to cover the axonal tracks thereby providing the appropriate niche for axonal metabolism and conductance. After introducing fluorescent markers to the glial cells, the movement of the glia is followed using confocal microscopy. The chain migration of the glia is unidirectional and starts at a narrow time window in development. With cell ablation technique it has been already shown that the first few cells in the chain, named pioneer cells, play a very important role in initiating and leading the migration. Upon elimination some or all of these first few cells, the migration of the chain is impaired (meaning delay in the completion of the migration or even complete stall). Now we would like to decipher the role and behaviour of these first cells in the chain. We carried out cell ablation experiments where we eliminated the glial cells in different numbers and in different combinations at the front of the chain. from these experiments we can conclude that the initiation of migration is not only dependent on the pioneer cells but also on the interconnection of these cells with the follower cells (i.e. with those that are behind the pioneers). The glial cells at the front of the chain require the vicinity of other cells: if we leave only a few cells intact in a cluster (i.e. less than 4-5 cells) by eliminating the neighbouring cells, the isolated cells are not able to move properly. The migration of the glial cells in a chain is a very complex phenomenon: while the first cells guide the rest of the chain towards the destination our recent findings indicate that these cells require the presence of the neighbouring cells. These findings may provide important information to the collective cell migration in cancer as well as in neurodegenerative diseases.
339/B286
TNFα and TGFβ Expression in Zebrafish Keratocyte Explant Cultures.
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Fish keratocytes are a useful, widely-used system to study cell motility. Typically, these cells are grown in primary explant cultures for 1-2 days before replating for use in cell studies focusing on individually migrating cells. Although it is a routine part of the experimental system, little is known about the initial explant culture period and any changes in gene expression during this period might affect keratocyte motility. The human analog of fish keratocytes, human keratinocytes, have a complex pattern of differential expression of cytokines and MMPs during primary culture. Therefore, we asked if similar changes in cytokine levels could be seen in the zebrafish keratocyte system and if these changes affect fish keratocyte motility. Initial data suggests that TNFa mRNA expression increases approximately 6 fold during the first 24 hours in culture and then decreases to values comparable to initial levels at subsequent time-points. In conjunction with data suggesting that TGFb expression by zebrafish keratocytes varies over time during explant cultures, we suggest that keratocytes may modulate the expression of cytokines during explant culture in a manner similar to the primary human keratinocytes and that expression of these compounds promotes fish keratocyte motility.

340/B287
Traction Force Mapping in Colliding Cells.
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The movement of fish epithelial keratocytes and other “fan” shaped cell types is unusual because these cells move in a direction that is perpendicular to the cell’s long axis. In keratocytes this mode of movement is associated with the existence of relatively large, inward facing traction stresses at the lateral cell edges. Preliminary data showed that the direction of keratocyte movement on an elastic substratum can be altered by stretching it on either side of the cell. To investigate whether keratocytes can respond to the traction stress generated by neighboring cells, we performed low magnification time-lapse imaging of colliding cell pairs, on elastic and glass substrata, for periods of 1-2 hours. On both surfaces, cells displayed similar responses that were characteristic of different types of collision. In general, the result of cell collisions is determined by the location and extent to which protrusion is inhibited by cell contact. However, a substratum dependent difference in behavior of colliding cells was seen when the front of one cell approached the lateral edge of the other. In these cases cells would begin to re-align with each other prior to making contact, and continue to move in the same direction after collision. Traction force microscopy of cells undergoing front to side collisions showed that regions of increased traction stress develop in both cells at sites of the cell margin that are within ~10 um of each other. As the cells reorient, regions of increased traction stress redistribute as the point of contact changes. Typically, the cell generating weaker forces will re-align, and appear to follow in the “wake” of the cell generating higher traction stresses. We term this phenomenon “tractotaxis” and conclude that this may provide short-range guidance cues to moving cells in vivo.

341/B288
A Critical Tension Abrogates Frictional Slip in Nascent Focal Adhesions.
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In directed cell migration, myosin-II tension impinged on lamellipodial, nascent adhesions modifies focal adhesion composition, signaling and size. To delineate the biophysical changes in focal adhesions during this maturation process, we utilized high resolution confocal and traction force microscopy to measure cytoskeletal dynamics and traction stresses simultaneously. Myosin-II-mediated focal adhesion maturation was synchronized by treatment and removal of the pharmacological inhibitor of myosin-II ATPase activity, blebbistatin. Myosin-II reactivation results in the dramatic shear slip between nascent adhesions and extracellular matrix (ECM) that is accompanied by rapid increase in cellular traction. This ‘frictional slip’ is concurrent with a notable
decline in F-actin retrograde flow that reaches a steady state around the time the focal adhesion position is stabilized. Subsequently, focal adhesions undergo elongation and F-actin bundles become apparent. Different classes of focal adhesion proteins, including vinculin, paxillin, talin and alphavbeta3 integrin, exhibit consistent, correlated slipping motion, indicating the interface of slip occurs at the integrin-ECM interface. Surprisingly, the magnitude and duration of this slip depends sensitively on the elastic properties of the extracellular matrix, with the largest amount of slip occurring on very soft matrices. By varying the stiffness of the ECM, we found that the stabilization of focal adhesions occurs over a narrow range of tension. By characterizing the changes in relative shear rates between the F-actin, focal adhesion, and ECM interfaces, we estimate that the effective viscosity between the integrin-ECM interface increases by several orders of magnitude during the myosin-II driven engagement of focal adhesions. In contrast, the viscosity of the F-actin-focal adhesion interface changes by less than an order of magnitude. Thus, the initial stages of myosin-dependent focal adhesion maturation are characterized by rapid strengthening of the integrin-ECM bonds under enhanced load to immobilize focal adhesions at a critical stress.

342/B289
Filamins Stabilize the Integrated Cytoskeleton.
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Cell motility is an essential, complex process that depends on a coherent cytoskeleton to physically coordinate the actions of numerous structural and signaling molecules. The cytoskeletal actin crosslinking protein, filamin (Fln), has been implicated in the support of three-dimensional cortical actin networks capable of both maintaining cellular integrity and withstanding large forces. However, the impact of simultaneous FlnA and FlnB loss on cell motility has not yet been elucidated. To this end, we used shRNA-mediated knockdown of FlnA in FlnB -/- mouse embryonic fibroblasts (MEFs) to produce severe depletion of Fln. In cell spreading on fibronectin-coated surfaces, Fln-depleted cells exhibited extensive ruffling of the cell edge, unstable adhesions, a condensed endoplasm, and transient traction forces on the substrate. Immunofluorescence confirmed defects in the stabilization of adhesion sites and actin stress fibers as well as defects in microtubule extension. Single-knockout FlnA -/- MEFs, but not FlnB -/- MEFs, also showed a significant defect in endoplasmic spreading, characterized by transient extension with abrupt retractions coupled with stress fiber breakage, identifying FlnA as the major player in the Fln-depleted phenotype. FlnA immunostaining and GFP-FlnA expression confirmed the localization of FlnA to regions surrounding the endoplasm. Our results implicate Flns in the stabilization of an integrated cytoskeleton and suggest that they also contribute to the spreading of the endoplasm.

343/B290
Distinct Functions of Anterior and Posterior Regions in Directional Cell Migration.
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Cell migration has generally been described as a cycle of coordinated steps: extension of the leading edge, adhesion to matrix, contraction of the cytoplasm, and detachment of the tail. However, neither the coordination of these steps nor the mechanism for moving the cell body has been clearly elucidated. We have addressed these questions by forcing cultured fibroblasts to take a linear shape with clearly defined anterior and posterior regions. A simple micropatterning method using linear polyacrylamide as the blocking agent was developed to control the cell shape and directional migration. When plated on narrow tracks, 3T3 fibroblasts show periodic tail retractions similar to cells in 3-dimensional matrices. Inhibition of Myosin II totally suppresses cell retraction but has little impact on cell protrusion at the leading edge, the morphology of the anterior region, or the translocation of the nucleus. Using GFP-a-actinin as a marker for the actin cortex, we observed distinct patterns of movement in different regions: stationary or slow forward transport in the anterior region, contraction and compression in the region immediately posterior to the nucleus, and stretching at the tail. Our results suggest distinct mechanical activities in
anterior and posterior regions during cell migration. Translocation of the anterior region is independent of Myosin II, while retraction of the posterior regions is strongly Myosin II dependent. Forces of the anterior and posterior regions converge in the middle, which may be responsible for positioning and forward movement of the nucleus during cell migration.

344/B291
The Effects of Force and Surface Stiffness on Breast Cancer Cell Migration.
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The transition of the mammary epithelium to a metastatic state is an important event that drastically increases mortality during breast cancer progression. Metastatic progression depends on the ability of tumor cells to migrate to nearby blood vessels and lymphatics before they can successfully metastasize and colonize into other tissues. An understanding of how biomechanical and biochemical signals influence directional migration to these sites of tissue escape could help develop therapies to slow or halt metastasis. To understand how the mechanical properties of the tumor microenvironment may affect metastasis, cell motility was measured by tracking mammary epithelia cells over night on fibronectin conjugated polyacrylamide gels of stiffness ranging from 140 Pa to 60,000 Pa as well as tissue culture glass. Average cell velocity and total distance traveled both increased with surface stiffness with maximal values observed at 2700 followed by a slight decrease in migratory behavior. Additionally, cells have been seen to respond to a surface stiffness gradient with directional movement towards the stiffer portion of the gradient. Mammary epithelia cells were tracked for 15 hours on fibronectin or collagen conjugated gels of gradient stiffness but uniform ligand density. Cells migrating on fibronectin gels showed significant directionality in the direction of greater stiffness while cells on the collagen gels did not. This suggests alternate migratory mechanisms utilized by mammary tumor epithelium when responding to the mechanical stimuli depend on fibronectin binding but not collagen. Both of these data underlie the importance of micro-environmental mechanical properties in dictating the migratory behavior of the tumor epithelium.

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Stem Cell Durotaxis: Implications for Trafficking to Rigid Fibrotic Scars.
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Scarring in the heart after a myocardial infarct, scarring in the skin after wounding, or scarring of the liver in cirrhosis - all lead to rigidification of tissue through extensive collagen crosslinking and can also lead to accumulation or ‘homing’ of adherent cells, including mesenchymal stem cells (MSCs). It is unknown how MSCs localize and, in particular, whether tissue rigidity plays a role. ‘Durotaxis’ is a term recently coined to describe the tendency in 2D culture for sparsely plated fibroblasts to crawl towards a stiff, collagen-coated gel made adjacent to a soft gel. Stiffness-induced motility would perhaps suggest cell homing to a stiff matrix, but clear evidence for accumulation of any cell type has been lacking. We cultured human MSCs on simple collagen-I coated gels with gradients in stiffness similar in magnitude to scars such as in the border zone of an infarcted heart. We first show that MSCs are indeed migratory with crawling speeds up to 1 micron/min. on stiffness gradients, we find that MSCs migrate toward the stiffer side of a substrate with definitive accumulation taking several days. Proliferation appears unaffected by stiffness in these systems, but to eliminate the potential for differential expansion, cell division was pharmacologically inhibited and durotaxis was once again documented. Consistent with past reports for cell spreading on homogeneous gels, the spread area of MSCs also increased along the stiffness gradient, which indicates that MSCs are not only responding to substrate stiffness but also tending to adhere more on stiff gels.
Amoeboid cell motility is a complicated process requiring the regulated activity and localization of many molecules and resulting in the cyclic repetition of a relatively small repertoire of shape changes. These changes are driven by the traction work produced by the cell, which can be estimated by measuring the forces and displacements exerted by the cells on their substrate during migration. We have developed and applied a novel implementation of Principal Component Analysis to identify and sort out the most important shape changes in terms of traction work produced by chemotaxing Dictyostelium cells. For this purpose, we acquired time-lapse recordings of cell shape and traction forces of Dictyostelium cells migrating on deformable substrates. Using wild-type cells as reference, we investigated the effect of altering Myosin II activity by studying Myosin II null cells and essential light chain null cells. Our results indicate that the spatio-temporal variation of the traction work produced by Dictyostelium cells can be described with a reduced number of modes. In fact, only four modes are needed to account for 65% of the traction work exerted by all cell lines studied. Furthermore, the first mode alone accounts for almost 50% of the traction work. Spatially, this mode consists of the attachment of the cell predominantly at two areas at front and back, contracting towards the center of the cell. The time evolution of this mode is approximately periodic and coincides with the time evolution of cell length. Each one of the remaining modes accounts for less that 10% of the traction work. Their temporal and spatial organization is less clear, suggesting that the cell performs a traction work cycle composed of a repetitive sequence of steps over which random fluctuations are imposed.

Human vascular endothelial cells (hVECs) serve as a critical barrier between the circulatory system and the rest of the body and participate in complex biological processes including blood transport, cell trafficking, and diapedesis. Failure of the vascular wall can result in serious and possibly life threatening complications. While vascular grafting using autologous and allogeneic material is routinely performed there remains a need for improved engineered vascular prosthetics. In the development of any solution, the influence of the three dimensional, nanoscale topography that exists In Vivo must be accounted for because of its influence upon cell behavior. To this end, we investigated the effects of biophysical cues on human umbilical vein endothelial cell migration. Previous studies have shown that native basement membranes that underlie the vascular endothelium possess feature sizes ranging from 400 - 4000nm. To simulate these features, polyurethane substrates containing either anisotropically patterned surfaces of parallel ridges and grooves or isotropically patterned surfaces with holes were produced that ranged in size from 400 - 4000nm in pitch (pitch = feature width + groove or hole width). Preliminary data demonstrate that migrating cells exhibit contact guidance on anisotropically patterned surfaces with the greatest rate of migration and distance travelled when cells were in contact with features of 1200nm or larger. Preliminary data suggest that on isotropically patterned surfaces the rate and distance traveled did not vary significantly from planar control surfaces. These data suggest that selected topographic features and size scales should be considered in the design parameters of vascular prosthetics.
Directed Cell Motility on 2-D Micron-Scale Patterns.
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Cell motility on 2-D surfaces has been extensively studied to unravel various cell-ECM interactions that affect cell migration. Recently, use of various micron-scale ECM patterns has shed light on the effects of geometrical constraint on cell survival and adhesion. However, limited work has been undertaken to study and manipulate cell motility using such patterns. Here we present various, micron-scale patterns based on asymmetric islands that can be used to control the directional bias and motility of epithelial cells. For example, we can program cells to move persistently in a counter-clockwise fashion around a path comprised of four asymmetric islands. We further demonstrate the ability to tune or alter these directional biases either by altering specific parameters of the micropattern or by altering intracellular signaling pathways by RNA interference. Finally, we apply these directional biases to partition cells between reservoirs connected by bridges composed of micron-scale patterns. Our findings provide new insights into how cells interpret micron-scale features and how such features may be used to direct cell motility over long length scales. These design principles provide a foundation for constructing “smart” devices for orchestrating multicellular organization, an essential building block for tissue engineering applications.

Mechanics in Neuronal Development.
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Nervous tissue consists of several different types of cells, blood vessels, and extracellular matrix. All these building blocks differ in their mechanical properties. Particularly during growth and migration, the local mechanical environment of neurons may thus change considerably. Here we show how neurons detect and avoid stiff substrates and how their mechano-responsiveness may be used to guide their axons along distinct pathways. In vitro, neurons continuously probe the mechanical properties of their environment. Growth cones visibly deformed substrates with a compliance commensurate with their own. To understand the growth cones’ sensing of stiff substrates, we investigated their precise temporal response to well-defined mechanical stresses. Externally applied mechanical stress exceeding the threshold of ~300pN/µm2 caused a calcium influx through mechanosensitive, stretch-activated ion channels in the growth cone membrane that triggered growth cone detachment and neurite retraction. Subsequently, neuronal processes re-extended, thereby enabling the exploration of alternative directions. To study the physiological consequences of this mechano-responsiveness, neurons were cultured on polyacrylamide gels of various compliances. Morphology, growth rate, and fasciculation of outgrowing retinal ganglion cell axons significantly depended on the mechanical properties of their substrate. On softer substrates, retinal ganglion cell axons fasciculated more and preferentially grew in a common direction, similar as in vivo, where these axons build the optic nerve. Hence, mechanics may be involved in neuronal development, leading axons together.

Strain Energy and Its Density Distribution around Invasive and Non-Invasive Carcinoma Cells in 3D Collagen Gels.
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Cell invasion through a dense 3D matrix is believed to depend on the ability of cells to generate traction forces. To quantify the role of cell tractions during invasion in a 3D reconstituted collagen
gel (shear modulus $G' = 118 \text{ Pa}$, 500 $\mu$m thickness, average mesh size 1.6 $\mu$m), we measured the
strain energy of highly invasive MDA-MB-231 breast carcinoma and A-125 lung carcinoma cells.
The results were compared to the strain energy generated by non-invasive MCF-7 breast
carcinoma and A-431 lung carcinoma cells. In all cases, cells locally contracted the gel. The
undeformed state of the gel was obtained after addition of the actin-disrupting drug cytochalasin-
D. Gel deformations were measured by tracking the 3D spatial positions of fluorescent beads (1
$\mu$m diameter) embedded in the gels. The bead positions served as nodes for a finite element
tessellation, from the local strain of each element and the known elasticity of the collagen, we
computed the local strain energy stored in the collagen gel surrounding the cell. As expected, the
strain energy of invasive breast carcinoma cells ($1.4 \pm 0.2 \text{ pJ}$, $n=34$) was significantly higher than
the strain energy of non-invasive breast carcinoma cells ($0.8 \pm 0.1 \text{ pJ}$, $n=28$). Surprisingly, the
strain energy of non-invasive lung carcinoma cells ($4.2 \pm 0.7 \text{ pJ}$, $n=31$) was similar to that of
invasive lung carcinoma cells ($3.5 \pm 0.4 \text{ pJ}$, $n=34$). Invasive cells, however, assumed an
elongated spindle-like morphology as opposed to the more spherical shape of non-invasive cells.
Accordingly, the distribution of strain energy density around invasive cells followed patterns of
increased complexity and anisotropy, quantified by its aspect ratio. These results suggest that not
only the magnitude of traction generation but also their directionality are important for carcinoma
cells invasion in 3D collagen gels.

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**Tumor Cell Invasion through Enhanced Contractile Force Generation.**

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The process of metastasis formation causes malignant progression of tumors. An important step
during metastasis formation is the migration of tumor cells into the extracellular matrix. The
migration velocity depends on mechanical and biochemical signals such as
adhesion/deadhesion, matrix degradation, cellular stiffness or fluidity, transmission and
generation of contractile forces. Here, we tested whether $\alpha_5\beta_1$-expression influences cell
invasion into dense 3-D collagen fiber matrices. We isolated $\alpha_5\beta_1$-high and $\alpha_5\beta_1$-low expressing
sub cell lines from parental MDA-MB-231 breast cancer cells and found that higher $\alpha_5\beta_1$
expression significantly (3-fold) increased cell invasiveness. Knock-down of the $\alpha_5$ integrin
subunit in $\alpha_5$-high cells leads to decreased tumor cell invasion. Biomechanical measurements
using fourier transformed traction microscopy revealed that the $\alpha_5\beta_1$-high expressing cells
generated 5-fold larger contractile forces. In agreement with this finding, cell invasiveness was
reduced after addition of the myosin light chain kinase inhibitor ML-7 or the Myosin II inhibitor
blebbistatin in $\alpha_5\beta_1$-high cells, but not in $\alpha_5\beta_1$-low cells, suggesting that the $\alpha_5\beta_1$
inTEGRIN expression enhanced cell invasion into dense collagen matrices through enhanced transmission
and generation of contractile forces.

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**Analysis of Live Cell Images to Determine Cell Population Migration Paramaters.**

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Stem cells are unique cells which have the potential to differentiate to multiple cellular lineages
[1], aiding in growth and regeneration of various tissues. Live Cell Imaging is a tool uniquely able
to define and capture stem cell activities, and monitor the behavior of a stem cell population in
culture, through numerous real-time measurements at the single cell level. This results in a
detailed behavioral phenotype of the stem cell population, which can be linked to In Vivo outcome
measures. However, collection of data is time intensive, and a hindrance to the analysis of large
sets of image data. This problem may be circumvented via an automated analysis system. The
quantitative data gained from LCI is a powerful way to characterize individual or populations of cells in a user specified environment. An analysis program, Image-Pro Plus, is currently used as the premier tool in obtaining numerous measurements of cellular behavior and growth characteristics. The time-lapsed image sequences [2] are imported into Image-Pro. from here, the software can separate cellular objects by color / contrast, and count individual objects. The resulting information may be exported by the user for further analysis. The calculated cellular parameters obtained here are routinely used in graphs to display the results, for comparison to values obtained in experimental methods. The opportunity to gather large amounts of data in a single experiment necessitates automation of the analysis process for feasibility. The actual number of measurements totals >12,000 per experiment when done manually. To achieve multiparametric analysis and data display in the form of graphs - the streamlining process becomes more complicated. Image-Pro allows for the implementation of Macros, or specified sets of instructions, in the form of code, to fine tune the computational process to the user’s needs. The more automated the overall process becomes, the easier it will be to gather and interpret the data, allowing for more experiments to be run or a more in depth look of each experiment may be obtained.

353/B300
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Chemotaxis, the chemically guided movement of cells, is important to many biological processes. Cells exhibit chemotaxis in response to external gradients of chemoattractants, which include soluble bio-molecules (such as cAMP for D. discoideum and Serine for E. coli) and dissolved gases (such as oxygen in bacterial aerotaxis). Chemotactic cells usually respond to temporal changes in the concentration of chemoattractant as well. We present three types of microfluidic devices for studies of different types and aspects of chemotaxis. Microfluidic devices of the first type generate linear and exponential concentration profiles of soluble factors with different slopes. Exponential profiles with the concentration of cAMP varying by 1.25%, 2.5%, 5%, 7.5%, and 10% over a distance of 10 μm (typical cell diameter) were used to study chemotaxis of D. discoideum.

We found that chemotactic response depends on both ambient concentration and steepness of cAMP gradient. A modification of the devices has a capability of switching between a stable exponential profile and zero chemoattractant in the test area in a time sequence mimicking the environment experienced by D. discoideum in a colony. Microfluidic devices of the second type expose adherent cells in a test area to a uniform concentration of attractant that can be changed it time in a broad range either step-wise, with ~0.5 s transition time, or in continuous linear time ramps, which can last between 10 and 800 sec. The devices were used in combination with fluorescence microscopy to test the molecular signaling responses of D. discoideum to step-wise temporal changes of cAMP concentration. The duration, latency, and magnitude of the responses was found to be dependent on the height of the steps. The microfluidic devices of the third type generate gradients of concentration of oxygen with a variety of shapes, including linear, exponential, and non-monotonic (wavy). As a proof of concept, the devices were applied to testing the response of B. subtilis to gradients of oxygen and cells were found to congregate at high oxygen concentrations.

354/B301
Cell Shape and Speed Oscillation in Migrating HL60 Cells and Neutrophils.
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It is frequently observed that migrating cells exhibit oscillations in shape and speed. Previous investigation in fish keratocytes has shown that fundamental modes of cell shape are closely correlated with global migration parameters, such as speed and persistence. In this study, we aim to study the oscillation of shape and speed in migrating neutrophils and HL60 cells, and the
correlations between them. We acquired time-lapse movie for over one hour with high temporal resolution on several migrating HL60 cells and neutrophils under a uniform field of chemoattractant. Cells were segmented frame-by-frame using automated image processing. Using principal component analysis of the obtained contour, we decomposed the variation of shape in migrating HL60 cells and neutrophils into few fundamental modes that are biologically meaningful. The first fundamental mode, representing elongation and roundedness, highly correlates with cell area and exhibits similar oscillatory pattern as cell speed and the protrusion-retraction cycle. The second fundamental mode resembles the turning phase of the cells. The two fundamental modes are observed in both HL60 cells and primary neutrophils, supporting the similarity of the two cell types. A more detailed analysis looking at temporal correlations of individual fundamental modes with global migration parameters (e.g., speed, angular velocity, acceleration, area speed) would help understand the mechanism of whole-cell integration during migration.

355/B302
The Mechanics of Cell Turning in Epithelial Keratocytes.
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The motility of fish epithelial keratocytes is typically highly directionally persistent, compared to other motile cells such as neutrophils or fibroblasts; but even for keratocytes cell trajectories are never perfectly straight. An open question remains as to what asymmetries along the left-right axis of the direction of cell motility arise to produce long-range cell turning events, which would be necessary for directed cell motions. In this study cell trajectories were characterized to quantify the amount of turning over time periods ranging from 2 minutes to 6 hours. An average individual keratocyte was found to exhibit approximately ninety degrees of rotation in traversing an average distance of 50 um over a time of 5 minutes. Cells could enter persistent states with high mean angular speeds yet low variance, producing circular tracks that retraced themselves. The magnitude of turning is an ergodic parameter, as an individual cell can transition between highly and poorly path persistent states. Cell shape was correlated to the magnitude of turning on the population level, and individual shapes become asymmetric during turns. Pharmacological inhibition of actin-myosin contractility inhibited cell turning, and conversely activation of myosin activity increased cell turning. Cells turned less at higher ambient temperatures, but other perturbations of actin motility or signaling pathways had no effect on turning. Cells can be forced to turn toward the cathode of an applied electric field (1 to 10 V/cm). Induced turns took ~5 minutes to complete and over-steering with a characteristic length scale (~30 um) was observed. Cells in a coherent turning event have small radii of curvature (~30 um) that produce up to a five fold speed differential along the leading edge. To produce higher speeds along the outer edge of the cell increased actin polymerization must occur on the side of the cell opposite the direction of the turn. This is correlated with an asymmetry in aspect ratio, actin distribution and myosin localization suggesting an organizational mechanism to maintain a persistent asymmetric state.

356/B303
Insights from Quantitative Cell Shape Analysis - Intermittent Protrusions and Internal Waves.
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Dynamic cell shape is a highly visible manifestation of the interaction between the internal state of a cell and its external environment. We quantitatively analyzed the dynamic change in shape of migrating Dictyostelium discoideum, a model system for the study of cellular migration. Using a snake algorithm, we extracted each cell boundary as 400 equidistant boundary points. By tracking each boundary point from frame to frame, we determine how measures associated with
boundary points, such as the local velocity and curvature of the boundary, change in time. To visualize and interpret the dynamic changes in cell shape we used modified kymographs or space-time plots, where space is measured along the boundary of the cell and color corresponds to the measure being visualized. This analysis shows that protrusions are intermittent and the direction of protrusions alternates in a zig-zag pattern, while retractions are sustained and straight. By comparing the spatial auto-correlation of the local motion, we found that extensions are more localized than retractions. From space-time plots of the local curvature, we see that localized protrusions originate at the front of cells and travel towards the back at a constant and reproducible speed of order ten microns per minute. We hypothesize that these localized protrusions of the cell membrane correspond to internal waves of actin polymerization and depolymerization. The speed and frequency of localized protrusions appears to exhibit greater inter-cellular variability than any effect induced by a wide range of surface chemistries, specifically glass, acrylic resin, bovine serum albumin, and gold nanoparticles. Having no surface, however, does appear to alter localized protrusions. The speed of localized protrusions on a cell whose front is protruding off of a tall cliff is greater than the speed of localized protrusions on cells migrating on surfaces.

357/B304
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Biosensors that report activation of native, unmodified proteins can help delineate complex cellular processes with minimal perturbation of normal behavior. However, sensors for endogenous proteins are rare, in part due to the absence of readily available binding reagents that are selective for the active form of the targeted molecule. To address this issue we combined high throughput screening of engineered state-specific binding elements with a fluorescence-based reporting system that turns these elements into biosensors. We have targeted Src family kinases (SFKs) since they are key signaling nodes that control numerous cellular functions, including migration and adhesion. Also, the multiple roles performed by Src kinases and their involvement in several signaling networks suggest spatio-temporally regulated pools of SFK activities. Phage display screening was used to generate fibronectin domain III (FN3) binders that selectively bind SH3 domains from Src family kinases. Pull down experiments demonstrated that an FN3 binds selectively to active Src kinases. Using merocyanine dyes developed in our lab for live cell imaging, we have converted this FN3 into a sensitive fluorescence-based biosensor for activation of Src family kinases. The new sensors reveals patterns of Src activation in migrating cells and in cells stimulated with growth factors. In migrating cells, a distinct band of Src activation was observed at the leading edge. This transient activation coincided with protrusion. We also observed precisely timed Src activation in linear and circular dorsal ruffles. In keeping with our overall aim of developing a generally applicable strategy of sensor design, we have generated multiple binders using HT screening and are developing biosensors specific to individual Src family kinases.

358/B305
Fluorescence Fluctuation Analyses Reveal That FAK-Paxillin and A5 Integrin-Talin Form Complexes within Adhesions.
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Transient, localized protein interactions are central to adhesion formation, turnover, and signaling during cell migration. In highly motile cells, initial contact with the extracellular matrix is established via nascent adhesions in the lamellipodium. These adhesions function as signaling hubs that regulate protrusion and adhesion and also serve as traction points for migration. Paxillin
and its putative binding partner FAK have emerged as critical regulators of migration. Using dual-color total internal reflection fluorescence (TIRF) and confocal imaging modalities, we measured the simultaneous fluctuations of paxillin and FAK fluorescence intensity and used cross-variance and cross-correlation techniques to detect their interactions and kinetics in living cells at high spatiotemporal resolution. The TIRF-based cross-variance analysis shows that paxillin and FAK reside and exchange as multimeric complexes in nascent adhesions. A phosphomimetic mutant of paxillin (Y31E, Y118E), which increases protrusion and adhesion assembly and turnover, significantly increases the complex size. In contrast, a non-phosphorylatable paxillin mutant (Y31F, 118F) that suppresses adhesion turnover reduces the paxillin aggregation nearly 2-fold. In addition, confocal-based cross-correlation analysis (ccRICS) showed only minimal levels of wild-type paxillin and FAK interactions in the cytosol; however, the phosphomimetic mutant forms robust, freely diffusing complexes. We also investigated the associations between talin and α5 integrin, two molecules thought to link the ECM to actin. We measured the differences between their rates of assembly using a high resolution derivative as well as cross variance analyses. Both analyses show that α5 integrin and talin reside in complexes in stable adhesions but enter adhesions independently. Taken together, our measurements demonstrate the feasibility of identifying and quantifying molecular complexes at high spatiotemporal resolution in adhesions in living cells. Moreover, our data reveal that two interacting pairs, paxillin-FAK and integrin-talin, appear to form complexes after they enter adhesions rather than entering as preassembled complexes.

359/B306
Spatio-Temporal Dynamics of RhoG Activation during Cell Migration.
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Background: Cell morphodynamics are critically important in a variety of normal and pathological processes, including neuronal cell migration in development, leukocyte transendothelial migration (TEM), and cancer cell metastasis. The Rho family GTPases are a class of molecular switches that regulate the cell cytoskeleton and cell migration. RhoG is a highly conserved but less well understood member of this family and plays a role in cell migration. Given that RhoG has been found to indirectly activate Rac1, we hypothesize that RhoG activation is involved in the initiation of cell protrusion at least in part by influencing the spatio-temporal dynamics of Rac1 activity. Testing this hypothesis requires measurement of RhoG activation and inactivation kinetics in real time. Methods: to test our hypothesis, we developed a new activation biosensor for RhoG, together with our published biosensor for Rac1 and computational approaches that allow us to resolve the coordination of Rho GTPases with submicron and seconds resolution. Using these techniques, we were able to relate RhoG and Rac1 activation locally to cell morphological dynamics, enabling us to determine the relationships between them and their effects on cell migration. Additionally, we have begun using this methodology to study leukocyte migration in a model of TEM. Results and Conclusions: RhoG is activated at the cell edge synchronous with edge advancement, whereas Rac1 is activated 2 μm behind the cell edge with a delay of 40 s relative to initiation of protrusion. Upon knockdown of RhoG, Rac1 activation shifts from 2 μm to 6.5 μm from the leading edge. These results demonstrate distinct roles at the leading edge for these GTPases. We have also found that Rac1 is activated with tight spatial and temporal control downstream of ICAM-1 engagement in a model of leukocyte TEM. This work was supported by NIH grants GM57464, GM008719, F30HL094020, and HL080166.

360/B307
Apocynin and Its Derivatives Disrupt the Signaling Pathway Leading to Metastasis of Cancer Cells.
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Cancer cells are characterized by the rapid, uncontrollable division and spreading of cells in the body. A drug that would suppress this process would prove to be very useful in the field of cancer
cell biology. The objective of this study was to show that Apocynin (4-acetovanillone), an NADPH oxidase inhibitor, inhibits the migration of cancer cells and to illustrate a mechanism by which it functions through a series of activity assays, such as Rac and Cdc42. Apocynin was the monomer used to synthesize Trimer Hydroxylated Quinone (HQIII), a more potent derivative of Apocynin, by enzymatic synthesis using soybean peroxidase enzyme. MTT proliferation and migration assays in a previous study illustrated a decrease in breast cancer cell metastasis when cells were exposed to Apocynin or HQIII at varying concentrations; similar results are obtained here in prostate cancer cells (DU-145). Filter-based migration assays demonstrate that cells treated with concentrations of Apocynin starting at 250 μM result in decreased cell migration. The more potent HQIII decreased cell migration at concentrations as low as .01 μM. The same concentrations had no affect on cell proliferation in the prostate cancer cells based on a MTT assay. These results indicate that Apocynin and it's derivatives could be a powerful anti-migratory compound for the treatment of cancer.

361/B308
Synthetic Triterpenoids Target Actin-Related Protein 3 (ARP-3) and Inhibit Cell Migration.
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Synthetic triterpenoids are anti-tumor agents that affect numerous cellular functions including apoptosis, growth inhibition, anti-inflammation and cytoprotection. Previously, an imidazolide derivative of 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO-Im) was shown to inhibit cell polarity and migration (To et al., J. Biol. Chem., 2008, 283: 11700-11713). The present study investigates the mechanism(s) whereby the synthetic triterpenoids inhibit cell migration. Using two proteomic approaches, protein array and mass spectrometry, we identified that Actin-related protein 3 (Arp3) and actin are triterpenoid binding proteins. Arp3, a subunit of the Actin-related protein 2/3 (Arp2/3) complex, is involved in branched actin polymerization and the formation of lamellipodia; processes which lead to cell migration. In-vitro pull down assays confirmed Arp3 to be a direct target of synthetic triterpenoids and immunofluorescence studies revealed that upon triterpenoid treatment, the localization of Arp3 and actin at the leading edge was inhibited. In-vitro branched actin polymerization was also observed to be inhibited by triterpenoids in a dose-dependent manner. Taken together, our data suggests that synthetic triterpenoids target Arp3 and branched actin polymerization to inhibit cell migration.

362/B309
Simultaneous Reconstitution of MSP-Based Protrusion and Retraction in the Amoeboid Sperm of Ascaris.
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Crawling movement in eukaryotic cells is produced by coordination of the force for protrusion of the leading edge and with that for retraction of the trailing cell body. In Ascaris sperm, protrusion and retraction are powered by a motility system comprised of major sperm protein (MSP) in place of the actin apparatus typically associated with cell migration. To explore the detailed mechanism of the MSP-based force production we developed an In Vitro motility system in which adjustment of the ATP concentration in cell-free sperm extracts resulted in the simultaneous reconstitution of protrusion and retraction in MSP filament meshworks called fibers. Under these conditions, polymerization of filaments immediately behind plasma membrane-derived vesicles to push the vesicle forward was accompanied by filament rearrangement and disassembly that pulled the rear of the fiber forward. This simultaneous assembly-disassembly resembled the dynamics of actin comet tails observed in Listeria and related In Vitro systems. Analysis of platinum replicas by EM showed a progressive loss of filament density from the front to the back of MSP comet tails. Interestingly, we also found that filaments in the retracting region of the tail exhibited alignment along the fiber axis that was not evident at the growing end of the tail. Thus, filament
orientation changes during fiber retraction. To explore this reorientation in real time we examined live MSP comet tails using a liquid crystal polarization microscope (LC-Polscope). We detected a chevron-like orientation of filaments at the newly assembled growing end of the comet tail that gradually converted to the axis-aligned pattern observed by EM as the comet tail disassembled and retracted. These data suggest that cell body retraction in *Ascaris* sperm is powered by the rearrangement and interaction of the subset of MSP filaments that survive depolymerization. Because no molecular motor proteins have been identified in the MSP motility apparatus the movement of filaments during MSP-based retraction appear to occur without the assistance of components analogous to myosin. Supported by NIH Grant R37 GM29994.

**363/B310**

**Rapidly Locomoting Fish Epithelial Keratocytes Switch Among Three Adhesion-Dependent Migration Regimes.**

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Keratocytes are fast-moving cells in which adhesion dynamics are tightly coupled to the actin polymerization motor that drives migration, resulting in highly coordinated cell movement. We have found that modifying the adhesive properties of the underlying substrate has a dramatic effect on keratocyte morphology. Cells plated on intermediate adhesion strength surfaces looked like stereotypical keratocytes, characterized by a broad, fan-shaped lamellipodium and persistent rates of protrusion and retraction. Cells plated on low adhesion strength surfaces were small and round with noisy protrusion and retraction rates, and cells on high adhesion strength surfaces were large and asymmetrical and, strikingly, exhibited traveling waves of protrusion. In addition, we imaged individual keratocytes as they crawled on micropatterned substrates, moving from regions of low to medium adhesion strength and from regions of medium to high adhesion strength. Individual cells were able to immediately transition among the adhesion-dependent behaviors observed in populations of cells, indicating that changes in adhesion strength of the underlying surface are sufficient to switch keratocytes between the three adhesion-dependent migration regimes. To elucidate the mechanisms by which adhesion strength determines cell behavior, we examined the size and distribution of adhesions and the organization and dynamics of the actin network in keratocytes migrating on substrates with different adhesion strengths. Our results suggest that on low adhesion strength surfaces, cell behavior is primarily dependent on the balance between adhesion forces and myosin contraction, and on high adhesion strength surfaces, cell behavior is primarily dependent on cycles of myosin-dependent maturation of adhesions, which periodically inhibit polymerization of the dendritic actin network at the leading edge, resulting in traveling waves of protrusion. on the whole, our results are consistent with a model in which keratocyte migratory behavior emerges from the self-organization of actin, adhesions, and myosin, and quantitative changes in either adhesion strength or myosin contraction switch keratocytes among migration regimes.

**364/B311**

**Characterization of a Cell Invasion Assay for High Content Screening.**

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The drug discovery process for novel anti-metastatic therapies depends upon robust screening assays that mimic 3-Dimensional tumor cell invasion through an extracellular matrix. We have utilized the 96-well Oris™ cell-based assay platform to perform 3-D invasion assays. Cells are seeded into wells coated with an extracellular matrix (ECM) such as basement membrane extract (BME) or type I collagen. These wells contain a silicone stopper that occludes cells from the well center and following cell attachment, the stoppers are removed to reveal a central exclusion zone that is void of cells. A second layer of ECM is overlaid onto the wells and cells are then permitted to invade into the exclusion zone. We have established the relative invasiveness of two widely
studied human cancer cell lines, HT-1080 fibrosarcoma and MDA-MB-231 breast carcinoma, using different concentrations of ECM overlays. Cells were fixed and stained with TRITC-phalloidin and DAPI followed by readout with the Acumen® eX3 high content microplate cytometry system. Results demonstrate that HT-1080 cells invade more aggressively than MDA-MB-231 cells into both BME and type I collagen gel overlays and that the amount of invasion for a given cell line is inversely proportional to the concentration of ECM used in the overlay. Additionally, use of the nuclear stain DAPI provides a more accurate quantitation of the number of invading cells compared to the F-actin stain. Our results suggest that 3-D invasion assays can be optimized for use with a variety of cancer cell lines by employing high content screening systems for data acquisition to generate Z’-factors suitable for compound screening.

**Germ Cells and Fertilization (365 - 384)**

### 365/B312
**C. elegans** FOG-3 Can Affect Germline Proliferation, Differentiation and Survival.

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FBF, FOG-1 and FOG-3 are key members of a regulatory network that controls germline fates in *Caenorhabditis elegans*.¹ FBF is a Puf RNA-binding protein, FOG-1 is a CPEB RNA binding protein, and FOG-3 is a Tob BTG homolog.¹ FBF regulates the expression of FOG-1 and likely FOG-3²; FOG-1 and FOG-3 are required for sperm fate specification and can influence germline proliferation,¹,² but less is known about how they drive those biological fates. This work focuses on our preliminary studies of FOG-3 and its control of germline fates. We first used genetic mosaics to confirm that FOG-3 controls the sperm/oocyte decision by acting within the germline tissue, as expected.¹ We next used a rescuing FLAG-tagged FOG-3 transgene to explore FOG-3 expression. FLAG-FOG-3 is seen in early meiotic germ cells destined to become spermatocytes, but it is no longer detectable in primary spermatocytes. That pattern is consistent with its role in sperm specification. FLAG-FOG-3 is primarily cytoplasmic, suggesting a post-transcriptional role. [In vertebrates, Tob BTG proteins have been implicated in both transcriptional and post-transcriptional controls.⁴] We are exploring FOG-3 dosage effects, in part because such effects were found for FOG-1.² Dosage effects are not seen in a wild-type background,⁵ but germlines in the progeny of *fbf(0); fog-3/+* animals can be spermatogenic, oogenic, deteriorated or tumorous. Our current experiments are focused on understanding how the dose of FOG-3 influences the network to produce these phenotypes. <P> <P>¹ Kimble J, Crittenden SL. Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in *Caenorhabditis elegans*. Ann Rev Cell Dev Biol 2007 23:405-33. <P>² Thompson BE, Bernstein DS, et al. Dose-dependent control of proliferation and sperm specification by FOG-1/CPEB. Development 2005 132:3471-81. <P>³ Chen PJ, Singal A, et al. A novel member of the Tob family of proteins controls sexual fate in *Caenorhabditis elegans* germ cells. Dev Biol 2000 217:77-90. <P>⁴ Jia S, Meng A. Tob genes in development and homeostasis. Dev Dyn 2007 236:913-21. <P>⁵ Ellis RE, Kimble J. fog-3 and regulation of cell fate in the germ line of *Caenorhabditis elegans*. Genetics 1995 139:561-77.

### 366/B313
**A Novel CRL4⁰DCAF1 E3 Ubiquitin Ligase Complex Is Critical for Ribosome Biogenesis and Nucleolar Morphology in C. elegans Germ Cells.**

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Cullin4 RING finger ubiquitin ligase (CRL4) complex is a key regulator of DNA replication initiation. Inactivation of CRL4 function produces a fully penetrant germ cell growth defect in nematode *C. elegans* although there is no evidence of DNA re-replication usually observed in
somatic cells. Notably, nucleoli in the mutant germ cells exhibit an abnormal, globular morphology. We identified DCAF-1 (DDB1 and CUL4-associated factor) as the substrate recognition subunit for the CRL4 germline function. The dcaf-1 mutant animals develop slower than wild type, and become sterile adults. We noticed that ncl-2 mutants have a similar germ cell nucleolar morphology defect implying that they are the same gene as dcaf-1 and ncl-2 mutants fail to complement each other. Transmission electron microscopy reveals that mutant germ cells possess fewer ribosomes than wild type, suggesting a profound defect in ribosome biogenesis. Recently we observed that reducing the activity of FOG-1, a cytoplasmic polyadenylation element binding (CPEB) protein, rescues the dcaf-1 germ cell nucleolar defect. We are currently investigating the role of CPEB proteins in ribosome biogenesis in order to better understand the molecular mechanisms underlying this novel function of the CRL4 DCAF1 complex in germ cell growth.

367/B314
Regulation of P Granule Integrity by the Phosphatase PP2A.
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Germ granules are ribonucleoprotein complexes found uniquely in germ cells. The mechanisms that hold these complexes together are not known. In the C. elegans zygote, germ granules are partitioned to the germline through a series of asymmetric divisions that separate soma from germline. During each division, P granules are selectively disassembled in the area of the cytoplasm destined for the somatic daughter cell, and are maintained and grow in size specifically in the area of the cytoplasm destined for the germline daughter cell. To gain insights into this process, we performed an RNAi screen for regulators of P granule integrity and identified protein phosphatase 2A (PP2A). Inactivation of the PP2A regulatory subunit caused P granules to disassemble throughout the cytoplasm during mitosis. As a result, P granule proteins were segregated equally into both somatic and germline daughter cells. Surprisingly, however, after division, P granules reappeared only in the germline daughter. Consistent with symmetric inheritance of P granule components, the P granules grew smaller and fewer with each division, eventually becoming almost undetectable. The embryos, however, developed normally and experienced only a partial loss of fertility. We conclude that asymmetric segregation of P granules during mitosis depends on the phosphatase PP2A, and is only essential for maximal fertility.

368/B315
Regulation of RNP Granule Assembly in Oocytes.
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As C. elegans hermaphrodites age, sperm become depleted, ovulation arrests, and oocytes accumulate in the germline. Large ribonucleoprotein (RNP) granules form in arrested oocytes that include: P granule proteins, P body proteins, and stress granule proteins. Large RNP granules also assemble in non-arrested oocytes when worms are exposed to environmental stresses such as heat shock and anoxia (1). We hypothesize RNP granules function in arrested or stressed oocytes to maintain RNA stability or prevent precocious translation until fertilization or a stress-free environment resumes. The somatic branch of the major sperm protein pathway appears to regulate the subcellular changes of RNPs in arrested or stressed oocytes (1). Our current goal is to better define the pathways regulating the assembly of the different classes of proteins that aggregate into large RNP granules in oocytes. We have undertaken a functional RNAi screen to identify genes required for RNP assembly when ovulation is arrested and identified 31 genes including: putative RNA-binding proteins, proteins involved in ribosome biogenesis, and several ATP and GTP-binding proteins including the C. elegans homolog of Dicer, dcr-1. In a candidate gene approach we have identified GLH-1 as a regulator of MEX-3, PGL-1, and CGH-1 assembly into granules. We have determined that GLH-1 and DCR-1 are mutually dependent by Western analyses, and that DCR-1 localizes to large granules in oocytes. Interestingly, MEX-3 ectopically localizes to large granules in young, dcr-1 worms, suggesting DCR-1 negatively regulates granule
assembly in young worms. Complementary TEM studies are aimed at further characterizing the ultrastructure of germline RNP granules both in arrested oocytes and in immature germ cells when granules are associated with the nuclear envelope. Preliminary analyses reveal the presence of free membranes with nuclear pores in arrested oocytes that are similar in appearance to annulate lamellae and an enrichment of nuclear pores clustered on nuclear envelopes at the position of RNP granules. 1) Jud et al., 2008. Dev Biol 318: 38-51.

369/B316
Polyadenylation of Stored Transcripts Mirrors Translational Patterns and Is Necessary for Proper Development during Spermatogenesis in Marsilea vestita.
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The regulated translation of stored mRNA is an important mechanism that underlies rapid development in a variety of organisms. The processing of stored transcripts is central in controlling rates and extents of rapid morphogenesis. The transcriptionally-quiescent process of spermatogenesis in the water fern Marsilea vestita offers an opportunity to study the mechanisms of post-transcriptional regulation and its role in rapid gamete development. The spermatozooids of Marsilea develop in an endosporic gametophyte that arises from a single meiotic cell contained within a microspore. Development begins with hydration of the microspore, which is followed by 9 rapid mitotic divisions cycles that produce 7 sterile cells and 32 spermatids. Each spermatid undergoes de novo basal body formation, cytoskeletal assembly, nuclear remodeling, elongation and coiling, and finally ciliogenesis, to form a motile male gamete. Throughout the 11 h process, little if any transcription occurs; instead development relies on the translation of stored transcripts and the utilization of new and stored proteins in gamete formation. We are interested in the role of polyadenylation in regulating the translation of stored RNA during spermatogenesis in the gametophyte. RNAi silencing and antibody localization assays were performed on homologs of the Cytoplasmic Polyadenylation Specificity Factor (CPSF100) and Cytoplasmic Polyadenylation Polymerase (PAP), respectively. CPSF100 silencing resulted in perturbed development, with the most severe effects halting gamete formation around 2 hours. from 2 hours of development up through full maturation, dramatic PAP accumulation was seen in the cytoplasm of spermatogenous but not sterile cells. The polyadenylation of stored transcripts was also assayed. Transcripts encoding the developmentally important homologs of Centrin, Magonashi, and SERK, all appear to undergo different levels of polyadenylation over time and these patterns presage the apparent levels of translation of each mRNA. These findings indicate that polyadenylation plays a key role in controlling the translation of stored transcripts in the male gametophytes of Marsilea vestita. Supported by NSF grants MCB-0720486 and DBI-0842525 to SMW.

370/B317
Characterization of Peroxiredoxin 3 Protein Expression in Drosophila melanogaster Testes.
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Peroxiredoxin 3 (Prx3) is a mitochondrial thioredoxin-dependent peroxidase that neutralizes excess peroxides, and helps protect against oxidative-stress-induced apoptosis. The gene DPX-5037 in Drosophila melanogaster encodes for a homolog of mammalian Prx3. DPX-5037 mRNA is expressed in all stages of Drosophila development. However, Prx3 protein expression in the Drosophila testes has not yet been established. We utilized immunofluorescence microscopy to examine the expression of Prx3 in the fly testis. Whole mounts and squashes were prepared from testes dissected from 0-3 day old flies. No signal was detected in control samples that were incubated without anti-Prx3 serum or heated anti-Prx3 serum. Signal was detected when samples were incubated with a 1:500 dilution of anti-Prx3 serum. Prx3 appears to be expressed in spermatogonia, spermatocytes, and round haploid spermatids. Prx3 signal is detected in the nebenkern as well as in the unfurling mitochondrial derivative. However, immunofluorescence did
not reveal any signal in the individualized sperm. Further studies using confocal microscopy will have to be performed to determine whether Prx3 is present in the tails of individualized sperm. In addition to establishing Prx3 protein expression in the adult fly testis, we also examined whether Prx3 is expressed in the larval testis. Whole mounts of testes dissected from wandering third instar larvae were prepared. Prx3 protein was detected in the larval testis. In summary, this study shows for the first time that the Prx3 protein is expressed in both larval and adult fly testes. Prx3 is expressed in the nebenkern and the unfurling mitochondrial derivatives. Further studies will be conducted to determine the role of Prx3 in Drosophila spermatogenesis.

371/B318
Auxilin Is Required for Proper Plasma Membrane Formation during Drosophila Spermiogenesis.
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In Drosophila, clathrin-mediated transport has been implicated in spermiogenesis, during which the haploid spermatids undergo drastic cell shape change to become functional sperms. It has been long appreciated that this transformation of spermatids from ovoid cells to long string-like cells requires extensive membrane biosynthesis and remodeling, although the precise mechanism remains unclear. To further understand the role of clathrin in this process, we have analyzed the phenotypes of viable Drosophila auxilin (dAux) mutations. As auxilin is known to participate in clathrin-mediated transport at multiple steps, dAux mutant males are sterile and produce no mature sperms. This reproductive defect of dAux males could be restored by germ cell-specific expression of dAux, indicating that, for spermatogenesis, auxilin function is required cell autonomously in the germ cells. EM analysis showed that in dAux mutant, the plasma membrane surrounding spermatids was not formed properly, resulting in the subsequent defect in investment cone (IC) migration. To understand the role of clathrin in spermatid elongation, we placed a GFP-tagged clathrin under the control of a germ cell-specific promoter. Using this reagent, we showed that, in wild type, intensely clathrin-positive vesicular structures were seen around the cell periphery and in the vicinity of the Golgi. In contrast, clathrin appeared mostly associated with enlarged Golgi in dAux mutants. Based on these results, we propose that during spermatid differentiation, clathrin mediates the membrane transport from the Golgi to the cell surface. In the absence of clathrin function (e.g. in dAux mutants), these Golgi-derived clathrin-dependent vesicles become reduced and the formation of the plasma membrane is deficient. Our data suggest that clathrin functions in both the endocytic and the secretory pathways during Drosophila development.

372/B319
Signaling Pathways in Activation of Water Strider Sperm Motility.
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Sperm are often maintained in a quiescent state and exhibit beating only upon activation by a signaling event. We are investigating the regulation of motility of sperm from the water strider Gerris remigis, which, like many insect species, produces extremely long sperm. The sperm flagellum of the water strider is ~2500 μm in length and can generate waveforms of varying amplitudes and frequencies. These sperm also exhibit a very unusual motility in which a region of the flagellum loops back upon itself and the two sections of the flagellum then rapidly twist around each other to form a tight helically coiled region. Sperm stored in the male seminal vesicles are normally immotile, but motility can be stimulated by addition of trypsin. We are investigating the signaling pathways that mediate activation of motility. The broad spectrum kinase inhibitor staurosporine completely blocked trypsin induced motility, and, conversely, the phosphatase inhibitor calyculin a was sufficient to activate motility. Sperm loaded with BAPTA-AM exhibited reduced levels of trypsin-induced motility, which was partially restored by the addition of A23187 and calcium. Incubation with A23187 and calcium alone partially activated immotile sperm, but to a lesser extent than calyculin A. These data suggest that a calcium sensitive component, potentially a kinase, mediates activation of motility. Preliminary results also suggest that several
sperm proteins are serine phosphorylated following trypsin activation. Trypsin has previously been suggested to activate sperm by degrading a glycoprotein matrix around the sperm, but treatment with PNGase F did not activate motility. Further, activation of motility by calyculin A alone suggests that degradation of a matrix is not required. Potentially, trypsin may activate a PAR2 type receptor on sperm to stimulate motility. Treatment of immotile sperm with thrombin, at levels of enzymatic activity equivalent to trypsin, did not activate motility, which is consistent with the behavior of the vertebrate PAR2 receptor. Characterization of this signaling cascade may reveal how sperm are maintained in a quiescent state until motility is activated.

373/B320
The Motility Lifetime of *Xenopus laevis* Sperm Is Extended by Egg Jelly-Derived Factors.
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*Xenopus* sperm motility is initiated by the osmotic shock experienced when these cells are delivered from the testes into low salt pond water. Motility is brief, a number of minutes, and is required for the sperm to penetrate the jelly layers and fertilize the egg. In this study we use video and time-lapse microscopy to demonstrate that egg jelly contains substances that prolong the motility lifetime of these sperm. The number of motile sperm in F1 (low salt) buffer decreased exponentially with a half time of about 3 minutes when viewed under cover glass. Addition of 10% v/v “egg water” (a jelly extract) increased the number of motile sperm about 2-fold over controls at 20 seconds and about 4- to 10-fold over controls between 5 and 10 minutes after initiation of motility. Egg water did not increase the average forward velocity of the sperm and the effect of egg water on motility lifetime was reduced but not eliminated in the absence of calcium (<10^{-7} M). Bovine serum albumin (20 μg/ml) and glucose (1 mM) did not produce this effect suggesting that it is not due to non-specific proteins or an energy source. Purified allurin, a 21 kD jelly protein known to be a sperm chemoattractant, did not produce this effect, indicating that other components of egg water are responsible. Motility of *Xenopus laevis* sperm and their response to egg water appeared to be seasonal with lower motility and response being observed in winter as opposed to spring. This study was supported by the NSF.

374/B321
Distribution of Cystatin Related Epididymal Spermatogenic (CRES) Protein in the Testis, Efferent Ducts and Epididymis and Alterations Associated with Its Absence in Cst8-/- Knockout Mice.
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CRES (Cst8) is a member of the cystatin superfamily of cysteine protease inhibitors. It differs from typical cystatins because it lacks consensus sites for cysteine protease inhibition and exhibits reproductive tract-specific expression. In the present study, we examined CRES expression within the testis, efferent ducts (EDs) and epididymis of normal mice by light microscope immunolocalization (LMI) following tissue fixation with zinc and by wide-field fluorescence imaging. Alterations to these tissues in Cst8-/- mice were also characterized by histomorphometry and electron microscopy (EM). In the testis of wildtype mice, CRES expression was restricted to the cytoplasm of elongating spermatids, while in the epididymis, principal cells showed intense supranuclear reactivity predominantly in the initial segment. The latter was suggestive of CRES secretion as also indicated by luminal reactions associated with sperm. In the cauda region, CRES appeared to be endocytosed by clear cells. In addition, distinct aggregates of CRES reactivity were noted in the epididymal lumen suggestive of its oligomerization. The presence of CRES mRNA by RT-PCR analysis in the EDs, along with the protein seen by LMI in the epithelium, suggests that the EDs synthesize CRES rather than endocytose it from the lumen. In older Cst8-/- mice, statistically significant reductions in tubular, epithelial and luminal profile areas of testis and epididymis were found compared to age-matched
wild type mice. Further analyses revealed significant differences by stages of the cycle in Cst8-/mice. In the EM, Cst8-/ mice showed vacuolated seminiferous epithelium, degenerating germ cells and premature release of germ cells into the epididymal lumen, which also contained abnormal sperm and the presence of large amorphous cocoon-like bodies. In addition, principal cells of the epididymis contained numerous large irregularly shaped lysosomes suggestive of lysosomal storage disease. These findings suggest that CRES is essential for testis and epididymal integrity and functions related to normal sperm maturation. (Supported by NIH HD56182 (GAC) and CIHR).

375/B322
Characterization of Rex1 (zf42) Knockout Mice.
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The Rex1 (zf42) gene has been identified by its reduced expression in F9 teratocarcinoma cells after retinoic acid treatment and is now considered one of the several stem cell markers in a complicated transcriptional network. Although the Rex1 gene has been studied extensively for several years, most of these studies focused on either its expression pattern or its interaction with other stem cell markers by chromatin immunoprecipitation assay. However, the function of Rex1 has not yet been thoroughly investigated. To study the function of Rex1, we created Rex1 knockout mice by homologous recombination followed by a conditional knockout using the Lox/Cre system. The results showed that the Rex1 knockout mice were not embryonic lethal and the ratio of male and female pups followed the expected Mendelian inheritance. Both male and female knockout mice were fertile. However, the fertility of male knockout mice seemed to decrease with age. The testis, which is the only adult tissue that has been shown to express Rex1, showed phenotypes followed. We found many vacuoles in the seminiferous tubules of Rex1 knockout mice and disruption of the edge of the tubules. Testes size was smaller in knockout mice as compared to normal, and sperm count was also decreased in the knockout male mice. TUNEL assay showed increased apoptosis in Rex1 knockout testes compared to wild type. Staining for the cell proliferation marker, PCNA, indicated abnormal cell proliferation and immunohistochemistry with specific cell markers, including androgen receptor, p450 scc, and dazL also showed abnormalities in Rex1 knockout mice. From these data, we conclude that the major function of the Rex1 gene may play a role in the process of spermatogenesis and may somehow affect Leydig cell function.

376/B323
The Blood-Epididymal Barrier Is Altered in Obstructive Azoospermic Patients.
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Azoospermia, defined as the absence of spermatozoa in the ejaculate, is present in 10 to 15% of infertile men and can be due to an obstruction of the epididymis. The epididymis is responsible for post-testicular sperm maturation. This maturation is dependent upon the creation of a specific microenvironment in the epididymal lumen which is partly created by the blood-epididymis barrier. This barrier is formed by apical tight junctions (TJ) between adjacent principal and principal/clear cells that line the epididymal lumen. Although several tight junctional proteins, including occludin and a large number of claudins (CLDNs), have been identified in the human and rat epididymis, their role and regulation in male infertility are relatively unknown. Our objectives were to determine if the blood-epididymis barrier was altered in obstructive azoospermic patients. In different regions of the epididymis of obstructive azoospermic patients, we observed by real-time PCR an altered expression of CDH1, TJP1, and CLDN1, 4, 7 and 10 compared to the same region of the epididymis of fertile patients. These results were confirmed by immunohistochemistry for CDH1 and TJP1. UsingIHCE1 cells, which are novel cell lines derived from the caput epididymidis of an obstructive azoospermic patient, we noted lower expression levels of several genes encoding junctional proteins (CDH1, CDH2, CLDN1, 3, 4, 7 and 8) than cell lines derived
from a fertile (FHCE1) patient. In addition, IHCE1 cells were not able to form tight junctions, as determined by measuring the transepithelial resistance (TER) across the cells, in contrast to FHCE1 cells. Microarray analyses comparing gene expression in IHCE1 and FHCE1 cells revealed differential expression of several genes encoding other junctional proteins and regulators of cellular junctions, such as RAB13, CDC42 and SNAI2. These results suggest that disruption of adherens, tight and gap junctions could therefore be involved in obstructive azoospermia and that the regulation of human epididymal TJs depends on the activation of specific intracellular signalling pathways. Supported by CIHR, FRSQ, and Armand-Frappier Foundation.

377/B324
Identification of Proteins Interacting with Adam7 in Sperm Membrane in Mice.
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In mammals, sperm acquire motility and an ability to fertilize eggs in the epididymis. This maturation process involves the acquisition of particular proteins from the epididymis. One of such secretory proteins is a disintegrin and metalloprotease 7 (ADAM7) expressed specifically in the epididymis. ADAM7 belongs to a family of transmembrane proteins, sharing a conserved multidomain structure: an N-terminal signal sequence, pro-, metalloprotease, disintegrin, cysteine-rich, EGF-like, transmembrane and cytoplasmic domains. Previous studies have shown that ADAM7 residing in an intracellular compartment of epididymal cells is transferred to sperm and the level of ADAM7 on sperm is dependent on the expression of ADAM2 and ADAM3 with critical roles in fertilization. In the present study, we investigated a molecular mechanism underlying interaction between ADAM7 and sperm. Immunoprecipitation with sperm lysates was performed using an anti-ADAM7 antibody and the resulting precipitates were analyzed by mass spectrometry. The proteomic analysis revealed that ADAM7 forms complexes with calnexin (Canx), heat shock protein 5 (Hspa5, a.k.a. glucose-regulated protein, 78 kDa) and integral membrane protein 2B (Itm2B). The interaction of ADAM7 with these proteins was confirmed by immunoprecipitation-western blot analysis. We found that ADAM7-interacting Canx, Hspa5 and Itm2B are located on the sperm membrane and, partly, in a detergent-resistant region in the membrane. Thus, our results suggest that the ADAM7 transfer from the epididymis to sperm occurs at the specialized domain on the sperm surface and ADAM7 functions in the fertilization process through the formation of the chaperonic complex.

378/B325
Meiotic Maturation Arrest Is Associated with Synaptic Disruptions in Human Zygotene-Pachytene Spermatocytes, Aberrant Sumoylation and Ubiquitylation.
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Spermatogenesis is highly susceptible to errors that result in reduction or failure in sperm production. Little is understood regarding underlying causes for meiotic arrest (MA) in men, while experimental genetic models have offered some insights regarding abnormal gene and protein expression. Using proteins extracted from human testes with normal spermatogenesis, we demonstrated that small ubiquitin-related modifier (SUMO) interactions occur during meiosis and synapsis, including SUMOylation of the synaptonemal complex (SC). To investigate whether faulty meiotic protein modifications could participate in MA, we isolated spermatocytes from biopsy materials obtained for possible testicular sperm extraction (TESE) for ART. Spermatocytes were obtained from men with obstructive azoospermia but normal spermatogenesis and those with non-obstructive infertility and MA, documented by histopathology. Fluorescent in situ hybridization (FISH), immunodetection and high-resolution bioimaging were performed. In an individual with MA, spermatocytes arrested at the zygotene-pachytene transition, with few observed to be in “true pachytene”; spermatocytes displayed prolonged asynaptic regions with possible non-homologous pairing. To identify whether double strand breaks (DSBs) were
resolved through meiotic recombination, spermatocytes were labeled with MLH1. MLH1 normally appears as small foci on SC throughout pachytene. In the MA patient, although there were no typical pachytene, MLH1 was identified, but with diminished intensity and greatly reduced foci. Aberrant SUMO-1 association was seen in zygotene-like cells with synapsed regions of incompletely synapsed chromosomes; normally SUMO-1 localizes to SC structures only after complete synapses. In MA, all prophase spermatocytes showed significant nuclear ubiquitin (Ubq) localization. In contrast, no ubiquitin (Ubq) was observed in spermatocytes from control samples at any stage of meiotic prophase. We demonstrate spermatogenic arrest at meiosis associated with aberrant SUMO-1 and Ubq in zygotene-to-pachytene transition spermatocytes, findings suggestive of a possible pathogenic role of SUMO-1 and Ubq in at least some patients with infertility.

379/B326
Molecular Basis of the Upregulation of Store-Operated Calcium Entry in Mammalian Maturing Oocytes.

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Calcium waves at fertilization trigger a number of cellular events that are required for the exit from metaphase II arrest and the resumption of the cell cycle in mammalian oocytes. We have suggested that store-operated calcium entry (SOCE) can be directly involved in Ca²⁺ signaling in mammalian oocytes at fertilization by regulating calcium influx. Upon ER depletion, the protein STIM1 which acts as Ca²⁺ sensor within the intraluminal space of this store, aggregates and activates a series of calcium channels at the plasma membrane, including ORAI1. However, the presence of ORAI proteins and its possible role on Ca²⁺ signaling has never been described in oocytes. On the other hand it is accepted that those mechanisms involved in the control of Ca²⁺ spikes are strictly optimized during meiotic maturation of oocyte, i.e. in the transition from immature germinal vesicle (GV) to mature oocyte (metaphase II or MII oocyte). After inducing meiotic maturation of mouse oocytes In Vitro we measured SOCE triggered by the treatment with 5 μM thapsigargin (TG) in Ca²⁺-free medium. We found that SOCE was absent in GV, whereas a minor SOCE was detected in GV breakdown stage (GVBD, prometaphase I). SOCE was found in intermediate levels in MI oocytes compared to the highest levels in MII oocytes. Although RT-PCR from total RNA using random primers showed similar levels of Stim1 transcripts in all stages of maturation, immunoblots revealed that STIM1 protein level is low in GV and that a sharp increase of expression occurs at the GVBD stage. Moreover, the relocalization of STIM1 triggered by the emptying of intracellular stores with TG, a marker of SOCE activation, was negligible in GV or GVBD, and limited in MI oocytes. Finally, we observed that Orai1 transcripts were present in all stages of maturation and that ORAI1 protein levels remained constant during this meiotic maturation. Taken together, our results suggest that STIM1 but not ORAI1 levels are limiting SOCE in immature oocytes and that the meiotic upregulation of STIM1 supports a fully response to ER depletion in MII oocytes. Supported by Junta de Extremadura (grant PDT08A027) and Ministerio de Ciencia e Innovación (grant BFU2008-00104).

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STIM1 Relocalization in Mouse Oocytes at Fertilization Suggests an Early Involvement of Store-Operated Calcium Entry.

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The transient and repetitive increases of the cytosolic free Ca²⁺ concentration are considered a crucial event in mammalian oocytes at fertilization. This signaling is essential for the exit from metaphase II arrest and the resumption of the cell cycle. This signaling is initiated by the sperm-specific phospholipase C (PLC) zeta with the production of inositol 1,4,5-trisphosphate, that binds
to its receptor at the endoplasmic reticulum (ER), leading to the release of Ca\textsuperscript{2+} from this intracellular store. In order to assess the contribution of store-operated calcium entry (SOCE) to this signaling we studied the localization of the protein STIM1 in oocytes under different experimental conditions. STIM1 has been described to recruit and activate store-operated calcium channels at the plasma membrane in other cell types. RT-PCR of total RNA isolated from oocytes, and immunoblot experiments show that STIM1 is expressed in mouse oocytes. Moreover, immunolocalization of the endogenous STIM1 demonstrate an ER-dependent distribution in clusters close to the plasma membrane in resting oocytes. Treatment of oocytes with 5 \( \mu \)M thapsigargin (TG) or 10 \( \mu \)M ionomycin leads to the rapid depletion of ER. In parallel, there is a significant redistribution of STIM1 over larger areas covering the periphery of the oocyte during this treatment, confirming that STIM1 senses calcium concentration within ER in oocytes. Since the pharmacological activation of endogenous PLC with \( m \)-3M3FBS induces a TG-like redistribution of STIM1 in the oocyte, we tested the hypothesis that SOCE could be involved in signaling at fertilization, a PLC-mediated process. We found that fertilization of oocytes leads to a significant and rapid relocalization of STIM1. In addition, short times of exposure of oocytes to sperm demonstrated that STIM1 relocalization is a very early event in fertilization, matching well with the first Ca\textsuperscript{2+} spike found at fertilization in mouse oocytes (i.e. 7-15 min after the exposure to sperm) suggesting that SOCE could be involved not only in the support of the long-lasting calcium waves, but also in the generation of the earliest calcium spikes. [Supported by Junta de Extremadura (grant PDT08A027) and MICINN (grant BFU2008-00104)].

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Identification of 14-3-3 Protein Isoforms and Their Differential Subcellular Distribution in Mouse Oocytes and Eggs.
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The 14-3-3 proteins are known regulators in important intracellular events including signal transduction, cell cycle control and embryonic development. 14-3-3 (also known as YWHA or tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein) plays a central role in mitosis in mammalian cells and meiosis in amphibians, but its role in mammalian meiosis has not been entirely defined. The mammalian isoforms of 14-3-3 are beta, eta, gamma, epsilon, tau/theta, zeta, and sigma (the official protein designation for 14-3-3 sigma is SFN). To begin an examination of the role of 14-3-3 in mammalian meiosis and oocyte maturation, we have determined which isoforms are present in oocytes and eggs and examined their subcellular distribution. 14-3-3 isoforms in protein extracts from oocytes and eggs were detected by Western blotting using isoform-specific antibodies. All isoforms were identified by Western blots of oocyte and egg proteins, except sigma. Immunofluorescence confocal microscopy confirmed the presence of all of the isoforms in both oocytes and eggs with characteristic differences in some of their intracellular localizations. For example, 14-3-3 epsilon is found throughout the oocyte with some cortical accumulation, while it is notably absent in the interior of the egg. On the other hand, 14-3-3 eta is diffusely dispersed in the oocyte and attains a uniform punctate distribution in the egg with marked accumulation in the region of the meiotic spindle apparatus. 14-3-3 tau, while distributed throughout oocytes and eggs, shows selective absence along the inner nuclear membrane of all oocytes examined. Some of the isoforms (beta, zeta and gamma) express prominent cortical accumulation in oocytes, but are uniformly dispersed within eggs. The identification and study of differential subcellular distribution of 14-3-3 isoforms in female germ cells will enable characterization of potential 14-3-3 isoform-specific interactions with other key proteins involved in meiosis and oocyte maturation. This will lead to a better understanding of the individual functional roles of the 14-3-3 protein isoforms in mammalian oogenesis.

382/B329
Strain Difference in Luteinizing Hormone Receptor Protein Expression in Mouse Ovaries.
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Induction of superovulation by gonadotropin treatment is widely used in reproductive experiments. However, our previous study (Reprod. Fertil. Dev., 8:975-80, 1996) demonstrated that the differential response among various mouse strains to gonadotropins still poses a major problem for practical applications. To elucidate the mechanism underlying the strain difference in superovulation induction, we planned qualitative and quantitative comparisons of the gonadotropin receptors in various mouse strains. At the previous ASCB meeting, we reported no difference in cDNA sequences and mRNA expressions of luteinizing hormone receptors (LHR) among mouse strains. In this study, we quantitatively compared time-dependent ovarian LHR protein expressions between high (BDF1) and low (A/J) responder strains at superovulation induction in mice. Five IU of equine chorionic gonadotropin (eCG) were injected into 4-week-old females. Ovaries were collected from females, four females per time point, 48, 51, 54, 57, 60, and 63 h after eCG injection. Membrane protein fractions of each ovary were extracted using ReadyPrep sequential extraction kits (Bio-rad), separated by SDS-PAGE, and transferred onto PVDF membranes (Pall). Ovarian LHR and α-tubulin (loading control) proteins were detected by Western blotting using SNAP i.d. (Millipore) with anti-LHR (Santa Cruz) and anti-α-tubulin (Rockland) antibodies. The blots were visualized with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and chemiluminescence (GE). The relative band densities of the two molecules were measured in a linear range with CCD-captured images (LAS-3000, Fujifilm). The amounts of ovarian LHR in both strains showed a similar bi-phasic change, but the first peak in A/J was 3-hour earlier (54h) than that of BDF1 (57h). The LHR levels reduced slightly after the peak and then rose again 63h after eCG injection. These differential peak times of ovarian LHR protein expression suggest that the strain-dependent timing for hCG injection might be considered for better superovulation induction. This work was supported by a grant from the Ministry of Health, Labour and Welfare of Japan.

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Ultrastructural Characterization of Porcine Oocytes.

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The knowledge of oocyte morphology is crucial to the development of reproductive biotechnologies associated to the female gamete, such as cryopreservation and In Vitro embryo production. The aim of the present work was to characterize the ultrastructure of pig oocytes in different developmental stages. Ovarian tissue and dissected antral follicles were processed for transmission electron microscopy, both routine and osmium-imidazole method (for lipid detection). Preantral follicles were classified as primordial follicles (one layer of flattened or flattened-cubical granulosa cells around the oocyte), primary follicles (one layer of cubical granulosa cells around the oocyte) and secondary follicles (two or more layers of cubical granulosa cells around the oocyte). Ultrastructurally preantral follicles presented an ellipsoid or spherical oocyte, with a homogeneous cytoplasm and oval nucleus. The most abundant organelles were round mitochondria, rough and smooth endoplasmic reticulum. At primordial follicle oocytes, lipid droplets had polarized localization. In primary follicle oocytes, lipids droplets were more dispersed in ooplasm. Junctions such as zones of occlusion and adhesion were present between oocyte and granulosa cells. Secondary follicles presented an oocyte with peripheral nucleus. Round mitochondria were organized as string of pearls. The zona pellucida was completely formed and some microvilli and granulosa cells projection ending in Gap junctions were seen through it. A lot of electron-lucent vesicles in ooplasm were observed. Along with round mitochondria and endoplasmic reticulum cisternae, antral follicle oocytes presented many electron-lucent vesicles all over the cytoplasm. When the osmium-imidazole method was preformed it revealed that most of the vesicles seen in secondary and antral follicles oocytes were lipid droplets. In conclusion, this work described the ultrastructure of pig preantral and antral follicles oocytes. Moreover, the use of osmium-imidazole showed that pig oocytes are rich in lipids and that lipid contents increases and changes in nature as the oocyte develops.
Lipid Activation of Src during Fertilization in *Xenopus laevis*.

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Based on fat blot, phosphatidic acid (PA) mass measurements and other data, our current model for Xenopus fertilization involves sperm activation of phospholipase D1b (PLD1b) which would increase PA, PA would then bind to and activate Src. As described in the work by K.-I. Sato, Src would then phosphorylate and activate phospholipase C which in turn would lead to the major event of fertilization: elevation of intracellular calcium. 1-Butanol (which inhibits PA production by PLD) or 2-Butanol (ineffective derivative) was incubated with Xenopus eggs for 90 minutes and, after insemination, Src activation was recorded (Western blot, Src tyrosine 418 phosphorylation). 2-Butanol (0.50%, 0.75%, 1.0%) treatment did not produce any decrease in the activation of Src at fertilization, while similar concentrations of 1-Butanol significantly reduced PP60Src. To quantify the rate of successful fertilization, videomicroscopy was used to measure the percent of cells undergoing gravitational rotation (GR). 1-Butanol (0.50, 0.75%) treated eggs did not undergo GR while 69% GR was recorded in 0.75% 2-Butanol treated eggs (untreated eggs: 100% GR). There was no change in total Src levels after butanol incubations (Western blot with a human Src antibody generated to the region of highest human and Xenopus Src homology). We also describe the release of intracellular calcium by addition of synthetic PA to Xenopus oocytes and report the I-V relationship for the calcium-activated chloride channels after PA addition.

Cell Polarity (385 – 408)

Bile Acids Regulate Bile Canaliculal Formation in Primary Hepatocytes.

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Bile acid synthesis and canaliculal formation are sparse in fetal hepatocytes, but increase significantly after birth. Based on these observations, we investigated the role of bile acids on canaliculal development using nondividing rat hepatocytes in collagen-sandwich cultures. Our previous study revealed association of canaliculal formation with activation of the LKB1-AMPK system. Methods: Immunofluorescent studies of tight junctions (occludin) and an apical ABC transporter (ABCB1) were used to characterize canaliculal formation. Phosphorylated/activated LKB1 and AMPK were quantified in Western blots. Results: Canaliculal formation revealed sequential morphologic patterns proceeding from a few small canaliculi (day 2) to an extensive branched network (day 6). Addition of taurocholate (25-200 μM), but not urso- or cheno-deoxycholate, on day 2 resulted in dose-dependent two-fold acceleration of canaliculal formation and activation of LKB1/AMPK. To understand the mechanism, pathways affected by taurocholate were studied. Inhibitors and/or activators of PI-3 kinase, CaMKK and FXR, which are activated by taurocholate, did not affect canaliculal formation. Since bile acids activate adenylyl cyclase and increase cAMP, we examined the role of adenylyl cyclase in taurocholate-induced canaliculal formation. Morphologic and LKB1-AMPK effects of taurocholate were reproduced by forskolin (100 μM), an adenylyl cyclase activator; prevented by adenylyl cyclase inhibitors (2’5’-dideoxyadenosine, 200 μM or MDL-12330A, 25 μM). Because taurocholate-induced canaliculal formation was cAMP-dependent, we studied two downstream cAMP-dependent pathways, PKA and Epac. Taurocholate-induced canaliculal formation was unaffected by PKA inhibition (14-22Amide, 500nM). In contrast, Epac activator (8-CPT-2’-O-Me-cAMP, 3 μM) produced the same effect as taurocholate on canaliculal formation and LKB1-AMPK activation. Inhibition of MEK, which is downstream of Epac, by PD98059 (100 μM) blocked taurocholate-induced canaliculal formation and activation of LKB1-AMPK. Conclusion: Taurocholate-induced canaliculal formation is mediated through adenylyl cyclase and cAMP, primarily through Epac downstream signaling, and is associated with LKB1-AMPK activation.
386/B333
Rab11a and Myosin Vb Are Required for Canalicular Network Formation in Rat Hepatocytes.
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The apical domain of polarized hepatocytes consists of an extensive canalicular network. Cytoskeletal elements, motor molecules and other components are proposed to participate in canalicular network formation; however, their specific functions are not known. Using collagen sandwich cultures of rat hepatocytes, we investigated the role of rab11a and myosin Vb, which are components of the apical recycling system, in canalicular formation. Freshly isolated rat hepatocytes were cultured on collagen-coated grass bottom dishes. After 24hr culture, cells were overlaid with collagen gel. One day after overlay with collagen gel, individual hepatocytes establish small circular canalliculi between adjacent cells. By day 3, canalliculi become tubular structures. Between days 3 and 6, tubular canalliculi fuse to form an extensive canalicular network. To study canalicular network formation, cells were infected with rab11aWT, rab11aS25N and myosin Vb tail adenoviral expression constructs at 24 hr after seeding. After 24hr expression of rab11aS25N and myosin Vb tail, individual canalliculi retained small circular morphology. at Day 6, infected cells fail to establish canalicular network. Expression of rab11aWT had no effect on the canalicular network formation. In exploring the basis for these effects, we monitored expression and distribution of endogenous canalicular membrane proteins, i.e. canalicular ABC transporters (ABCB1, ABCB11, ABCC2). Canalicular ABC transporters are minimally expressed at Day 1. The amount of canalicular ABC transporters progressively increased after collagen overlay. at Day 6, endogenous canalicular ABC transporters localize on rab11a-positive endosomes. Expression of rab11aS25N perturbed expression of canalicular ABC transporters. Live cell imaging revealed that rab11aS25N expression inhibited constitutive cycling of ABCB11-YFP. These results suggest that rab11a and myosin Vb play essential roles in canalicular network formation by regulating membrane traffic to canalicular domain.

387/B334
Mathematical Model of Dual Mechanisms of Cdc42 Recycling in Yeast: Relationship of Internalization Rate and Morphology.
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Biological processes such as development, cell motility, and immune response depend on the maintenance of robust cell polarization. Cdc42, a conserved Rho-GTPase, is a master regulator of cell polarization. Previous studies in yeast have found that after initial polarity establishment, the individual molecules of Cdc42 are rapidly exchanged between the membrane and the cytosol. Despite these rapid dynamics, the site of Cdc42 accumulation is held constant to allow for polarized growth. Here, we provide evidence that, after initial symmetry breaking, Cdc42 is dynamically maintained at the polar cap by recycling between the membrane and intracellular regions by two independent mechanisms: actin-mediated transport / endocytosis and cytosolic recycling through the Rho-GDP dissociation inhibitor (GDI) Rdi1. However, the mechanism by which the spatial relationship of these dual recycling pathways is controlled is unclear. Here, we present the results of a study combining live cell fluorescence measurements with a detailed mathematical model to examine how dual recycling pathways of Cdc42 in yeast work together to shape the Cdc42 membrane distribution. We find that in order to recapitulate the steady-state distribution of WT Cdc42, the dual recycling pathways must employ overlapping delivery windows of similar size. Interestingly, we find that Rdi1-mediated tuning of a single dynamic parameter, internalization rate inside the delivery window, is sufficient to explain differences in the Cdc42 cap distribution between cells undergoing pheromone response and cycling cells progressing to bud emergence. These changes in Cdc42 cap distribution are correlated to the observed morphogenic differences under these conditions. We find that the mechanism of action of each recycling pathway relies on the ability of Cdc42 to cycle between nucleotide bound states, and
does not depend on the states themselves. In addition, we employed fluorescence cross-correlation spectroscopy (FCCS) in live yeast between Rd1 and Cdc42 and Cdc42 mutants to provide insight in cytosolic interactions that are involved in the mechanism of Rd1-based recycling.

388/B335
The Exocyst and the Par-Cdc42 Complex Regulate Ciliogenesis.
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Our goal is to understand the role of the highly conserved eight protein exocyst complex in ciliogenesis and cystogenesis. Cysts are building blocks for epithelial organs such as the kidney, and defects in cyst formation lead to polycystic kidney disease (PKD). We previously showed that the exocyst localized, in renal cells, to the primary cilium, an organelle involved in flow sensing and PKD pathogenesis. We also showed that the exocyst was centrally involved in ciliogenesis. Most recently, we showed that exocyst protein Sec10 regulates primary ciliogenesis, with shRNA knockdown of Sec10 leading to decreased ciliogenesis and cystogenesis, and Sec10 overexpression leading to increased ciliogenesis and cystogenesis. In addition, the exocyst and Par3, which is also necessary for ciliogenesis, co-localized and co-immunoprecipitated (Zuo et al, 2009, Mol Biol Cell). Cdc42 is part of the Par complex, and we show here, using tetracycline repressible cell lines, that expression of dominant negative Cdc42 prevents ciliogenesis and leads to abnormal cystogenesis with inverted polarity. Interestingly, expression of constitutively active Cdc42 also decreases ciliogenesis (though only by 50%) and also leads to inverted cyst morphology. Similar to PKD cells, Fura 2-loaded Sec10 knockdown cells exhibited a reduced basal Ca\textsubscript{2+} concentration that did not respond to increases in apical flow, while cytosolic Ca\textsubscript{2+} concentration in the Sec10-overexpressing cells was higher and these cells exhibited Ca\textsubscript{2+} transients with increased apical flow. Again similar to PKD cells, the Sec10 knockdown cells were hyperproliferative. Finally, we show In Vivo correlation with injections of an antisense Sec10 morpholino in zebrafish. Sec10 morphants shared many phenotypes with pkd2 morphants, including: A curly-up tail, brain defects, and cystic dilation of the pronephric region. These data support a model in which the Par-Cdc42 complex localizes the exocyst to the primary cilium, and the exocyst then targets and docks vesicles carrying proteins necessary for ciliogenesis. When this does not occur, ciliogenesis and cystogenesis are defective.

389/B336
Dynamin Participates in the Maintenance of Anterior-Posterior Polarity in the C. elegans Embryo.
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The PAR polarity factors and the actin cytoskeleton are crucial for generating cell diversity throughout development. Recent evidence from a genome-wide RNAi screen in C. elegans reveals that anterior polarity cues (PAR-6, PKC-3, PAR-3, and CDC-42) can also specifically regulate the endocytic machinery. Current models for the role of PAR proteins in polarity suggest that PAR-dependent membrane recycling is required to generate or possibly maintain a plasma membrane domain and boundary, yet the links between the PAR proteins, endocytic machinery and the actin cytoskeleton during development have not been identified. Dynamin is a large GTPase that plays key roles in both endocytosis and actin dynamics and therefore a possible factor that could connect these events to cell polarity pathways. Here, we show that C. elegans dynamin, DYN-1, is required to maintain the anterior polarity factors PAR-6, RHO-1 and CDC-42. Interestingly, DYN-1-GFP foci are enriched in the anterior cortex of the embryo where endocytosis primarily occurs. DYN-1 regulates anterior membrane and actin comet dynamics specifically during the polarity maintenance phase. Foci of actin closely associated with RAB-5 foci, suggesting a link between actin-based endocytosis in the one-celled embryo. Finally, PAR-6-
labeled foci are closely associated with DYN-1-GFP foci and with endosomal markers, suggesting that PAR-6 is endocytosed and possibly recycled back to the anterior cortex during maintenance phase. Our results demonstrate a dynamin-dependent mechanism for the spatial and temporal regulation of polarity, endocytosis and actin dynamics in the anterior of the embryo, contributing to the precise localization and maintenance of polarity factors within a dynamic plasma membrane. Currently, we are interested in further characterization of the links between membrane trafficking and anterior-posterior polarity in the embryo.

390/B337
Cdc42 and Par Proteins Stabilize Dynamic Adherens Junctions through Interactions with Membrane Trafficking Proteins.
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Cdc42 and the Par complex are part of the intricate network of factors that establish and maintain epithelial cell polarity. Cdc42 binds to Par6 to promote the kinase activity of atypical protein kinase C (aPKC), which supports formation of the apical membrane. When Cdc42 function is compromised in Drosophila embryos, apical proteins are lost from the plasma membrane and instead accumulate in enlarged endosomes. Using the epithelium of the Drosophila embryonic neuroectoderm as a model, we found that this redistribution of apical membrane components is accompanied by loss of adherens junctions (AJs) during the ingression of neural progenitor cells, a process requiring extensive cell rearrangement. Our data support the hypothesis that the loss of AJs during cell rearrangement is a downstream consequence of the depletion of important polarity factors, such as Crumbs, from the apical membrane. Par complex mutants also accumulate apical markers in endosomes. Furthermore, Cdc42 mutant defects are rescued by expression of an activated form of aPKC. These results suggest that Cdc42 and Par proteins function in the trafficking of apical proteins, and that aPKC kinase activity is the key effector mechanism. Our current results indicate that Cdc42/Par interact genetically with genes encoding proteins involved in endocytosis and recycling, including components of the early endosome, ESCRT and retromer machinery. We will discuss our efforts to identify direct targets for aPKC phosphorylation amongst these trafficking proteins.

391/B338
Theoretical and Experimental Study of Par Protein Polarization in the Early Caenorhabditis elegans Embryo.
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Par proteins establish complementary domains in the early Caenorhabditis elegans embryo, polarizing the cell. It has been demonstrated that Par proteins interact through mutual phosphorylation, and in Drosophila melanogaster, it has been shown that PAR-3 oligomerization is required for proper localization of the Par proteins. The objective of this study is to determine whether these known interactions between Par proteins can lead to stable maintenance of the Par domains in the early embryo. Theoretically, we develop a mathematical model consisting of coupled differential equations whose terms use first order, mass action kinetics to describe interactions between the Par proteins. We find that mutual phosphorylation alone is not sufficient for maintenance of Par protein domains in the early embryo. We demonstrate that PAR-3 oligomerization along with mutual phosphorylation is sufficient to maintain complementary domains. The model predicts a sudden loss of polarization as a result of Par protein depletion. We test this experimentally, using epifluorescent microscopy of live transgenic embryos expressing fluorescent proteins to observe the localization of Par proteins, and RNAi to modulate protein expression. We find that experimental tests are consistent with behaviors predicted by the mathematical model. The model can explain previous observations, such as the possible mechanism behind Par protein mislocalization when the PAR-3 oligomerization domain is impaired, and how polarization may be rescued when both anterior and posterior Par proteins are sequentially or simultaneously depleted. The model also predicts possible cues for the initiation of Par protein polarization in the early embryo.
392/B339

**Generation of Asymmetry in the Cytoplasm by Par-1-Dependent Local Regulation of Protein Diffusion.**

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The PAR polarity regulators promote cell polarity by creating distinct domains in the cortex and cytoplasm of cells. How PAR asymmetry at the cortex promotes protein asymmetry in the cytoplasm is not understood. Here we report that PAR-1 polarizes the distribution of the cytoplasmic RNA-binding protein MEX-5 by directly phosphorylating MEX-5 and locally increasing its diffusion rate. During polarization of the C. elegans zygote, PAR-1 becomes enriched on the posterior cortex. The cell fate determinant MEX-5 accumulates opposite PAR-1 in the anterior cytoplasm. Segregation of MEX-5 requires PAR-1, but the mechanisms involved were not known. Using a photoconvertible Dendra2 tag and live imaging, we have found that before polarization, MEX-5 diffuses quite slowly throughout the cytoplasm (~1um²/sec). During polarization, MEX-5 diffusion increases specifically in the posterior (to ~3um²/sec). As a result, MEX-5 levels increase in the anterior, where the diffusion rate remains slow. Similar observations were made by Tenlen et al., 2008 using FRAP analysis. What increases MEX-5 diffusion in the posterior? In vitro, PAR-1 phosphorylates MEX-5 directly on two residues: S548 and S404. We found that S404 is phosphorylated In Vivo and is absolutely essential for MEX-5 asymmetry. A MEX-5(S404A) mutant remains slow diffusing and uniformly distributed during polarization of the zygote. Loss of par-1 kinase activity also slows down MEX-5, whereas ectopic localization of PAR-1 increases MEX-5 mobility throughout the cytoplasm. Interestingly, mutations that change MEX-5’s affinity for mRNA In Vitro (Pagano et al, 2007) also increase MEX-5 mobility. Together these results suggest the following model: 1) MEX-5 mobility is slowed by a uniformly distributed RNA anchor or complex, 2) direct phosphorylation by PAR-1 releases MEX-5 locally in the posterior cytoplasm, 3) As MEX-5 diffuses away from PAR-1, de-phosphorylation traps MEX-5 dynamically in the anterior cytoplasm. Our observations provide a simple model for how a cortically-enriched kinase can localize proteins in the cytoplasm by local regulation of protein diffusion.

393/B340

**Spatiotemporal Regulation of the Membrane Cortex in Cleavage Stage Sea Urchin Embryos.**

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Previously, we have shown that the polarity proteins of the Par complex as well as the membrane raft marker ganglioside GM1 are localized to the free, apical surface of cleavage stage sea urchin embryos as early as the two cell stage. We investigated the spatial and temporal regulation of this polarity using Fluorescence Recovery After Photobleaching (FRAP) analysis and immunofluorescence in one, two, and four cell stage sea urchin embryos. GM1 was found to be mobile only during late anaphase in control embryos. Disruption of actin or microtubules via treatment with Cytochalasin D, Latrunculin B, or Nocodazole had little effect on GM1 mobility in the apical membrane. When Myosin II phosphorylation was inhibited by treatment with the myosin light chain kinase (MLCK) inhibitors ML-7 and ML-9, or the Rho-kinase (ROCK) inhibitor H1152, GM1 was found to be mobile regardless of the cell cycle stage, and this disruption was enhanced in dissociated blastomeres. Immunofluorescence analysis revealed changes in myosin distribution from being predominantly apical to near even distribution around the membrane cortex. When Myosin II phosphorylation was inhibited by treatment with the myosin light chain kinase (MLCK) inhibitors ML-7 and ML-9, or the Rho-kinase (ROCK) inhibitor H1152, GM1 was found to be mobile regardless of the cell cycle stage, and this disruption was enhanced in dissociated blastomeres. Immunofluorescence analysis revealed changes in myosin distribution from being predominantly apical to near even distribution around the membrane cortex. Upon dissociation of blastomeres. Inhibition of myosin phosphorylation by MLCK also affects the apical localization of the Par complex proteins Cdc42, Par-6, and atypical protein kinase C (aPKC) as seen by immunofluorescence. These studies suggest that myosin filaments are involved in the spatial regulation of the membrane cortex as well as its temporal control during anaphase in early sea urchin development.

394/B341

**Dissecting the Role of Individual PDZ Domains in Bazooka/Par-3 Cortical Localization and Clustering in Drosophila.**
The scaffold protein Bazooka (Baz/PAR-3) functions in positioning adherens junctions (AJ)s in the developing Drosophila embryo. Baz contains three PDZ domains, which are protein-protein recognition modules known to organize multiprotein complexes. Recently we showed that Baz and AJ components form clusters independently, and that Baz clusters may act as molecular nets to aggregate AJ clusters at AJ assembly sites. However, the mechanism of how Baz clusters are localized, formed and maintained at the cortex is not understood. To determine the role of Baz's three PDZ domains in its cortical localization and clustering, we generated transgenic flies expressing Baz with no PDZ domains, with each domain alone and with each possible pair of domains. Each construct is tagged with GFP and located at the same chromosomal site. Using the UAS-GAL4 system, we expressed and analyzed them with 3-D live spinning disk microscopy with/without endogenous Baz in the lateral epidermis at dorsal closure. Endogenous Baz shows an overall decrease in cortical levels, preferentially along the A-P contact leading up to dorsal closure. At dorsal closure, removal of all PDZ domains lead to a more even cortical localization compared to full length Baz, but at comparable levels. Surprisingly, the addition of PDZ1 reduced cortical levels. In contrast, adding PDZ2 or PDZ3 elevated cortical levels above those of the full length construct. Remarkably, when PDZ1 is added back with either PDZ2 or PDZ3, normal cortical levels were restored. Taken together, these results suggest PDZ1 mediates the removal of Baz from the cortex. We find that elevated levels of Baz lead to greater clustering at the cortex. Moreover, the excess Baz in the absence of PDZ1 preferentially accumulated along A-P contacts.

We hypothesize that the correct localization of Baz relies on a balance between anchorage at the cortex and removal, with the former mediated by PDZ2 and PDZ3 and the latter mediated by PDZ1.

Selective Targeting of ER Exit Sites Supports Axon Development.
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During neuron development, the biosynthetic needs of the axon initially outweigh those of dendrites. However, while a localized role for the early secretory pathway in dendrite development has been observed, such a role in axon growth remains undefined. We therefore studied the localization of Sar1, a small GTPase that controls ER export, during early stages of neuronal development that are characterized by selective and robust axon growth. At these early stages, Sar1 was selectively targeted to the axon where it gradually concentrated within varicosities in which additional proteins that function in the early secretory pathway were detected. Sar1 targeting to the axon followed axon specification and was dependent on localized actin instability. Changes in Sar1 expression levels at these early development stages modulated axon growth. Specifically, reduced expression of Sar1, which was initially only detectable in the axon, correlated with reduced axon growth, while over-expression of Sar1 supported the growth of longer axons. In support of the former finding, expression of dominant negative Sar1 inhibited axon growth. Thus, as observed in lower organisms, mammalian cells utilize temporal and spatial regulation of ERES to address developmental biosynthetic demands. Furthermore, axons, like dendrites, rely on ERES targeting and assembly for growth.

Noncanonical Frizzled Signaling Regulates Cell Polarity of Growth Plate Chondrocytes.
Y. Li, A. T. Dudley; Northwestern university, Evanston, IL

A central question in developmental biology is how morphologically distinct structures are generated from similar cell types. The skeleton provides a good model system to answer this question due to the highly diverse and complex morphological differences displayed by individual elements. Analysis of the cartilage growth plate by Dodds suggested that longitudinal growth of long bones might result from the specific arrangement of chondrocytes. In the proliferative zone,
chondrocytes become discoid and arrange in columns, like stacks of coins, which are parallel to the long axis of the cartilage. Although well-organized chondrocytes derive from a relatively unstructured pool of progenitor cells, it is not known whether this arrangement of cells is the result of a regulated process or is in response to physical constraints of the cartilage matrix. We test this model that cell columns in the growth plate cartilage form from a single progenitor cell by a process in which cells divide orthogonal to the stack then rearrange. Our work has uncovered a central role of noncanonical Frizzled dependent signaling in the regulation of chondrocyte polarity and morphogenesis in the growth plate cartilage.

397/B344
Structural Analysis of the Interactions between PIP3-Binding Protein Centaurin A1 and the FHA Domain of Kinesin-3 Motor Protein Kif13b.
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Phosphatidylinositol (3,4,5)-trisphosphate, PIP3, is an important second messenger for the differentiation and development of neuron axons. The PIP3-binding protein centaurin α1 (CENTA1) contains one ArfGAP domain and two pleckstrin homolog (PH) domains in tandem. The CENTA1 is involved in the transport of PIP3-containing vesicles by interacting with the forkhead associated (FHA) domain of KIF13B (KIF13B-FHA). We solved the structures of CENTA1 in complex with PIP3 and of CENTA1 in complex with the KIF13B-FHA. The KIF13B-FHA interacts mainly with the first PH domain of CENTA1 using a novel interface that does not include a phospho-threonine. We verified the specific interactions between CENTA1 and KIF13B-FHA using a GST-pull down assay and isothermal titration calorimetry. The PIP3-bound CENTA1 structure suggested the second PH domain of CENTA1 could bind phosphatidylinositol (3,4)-bisphosphate, Ptdlns(3,4)P2, as well as PIP3, as confirmed by a PIP-strip assay. The structural results presented here are the first three dimensional model of a kinesin cargo binding domain complexed with a cargo adaptor protein, and the interaction of CENTA1 with PtdIns(3,4)P2 proposes a new physiological function of CENTA1 that can transport PI(3,4)P2-containing vesicles.

398/B345
Investigating the Role of Abnormal Spindle Protein Asp in Brain Development.
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Drosophila melanogaster Abnormal Spindle Protein (Asp) was first identified by phenotypic characterization of a late larval-pupae lethal mutation that display defects in spindle morphology, mitosis and meiosis. The gene encodes a 220 KDa microtubule-associated protein that contains actin and calmodulin binding domains. Interestingly, the human homologue of Asp, ASPM (abnormal spindle-like microcephaly-associated), is one of the genes implicated in primary microcephaly, a congenital disorder characterized by reduced brain size, specially the cerebral cortex, with no other abnormalities either within or outside of the nervous system. In addition, the mouse homologue is known to be necessary for the symmetric divisions of neuroepithelial cells during brain development. Thus, Asp function likely resides in the regulation of the proliferation of neural progenitors during neurogenesis. We utilized the larval Drosophila brain as a model system to ascertain the role of Asp during neurogenesis. Analysis of the dynamics of the protein In Vivo showed that Asp displays a cell cycle-dependent localization. However, during mitosis, it specifically localizes to spindle microtubules and spindle poles. In the larval mutant brain, as expected, several mitotic abnormalities were observed. The dividing neural progenitor cells or neuroblasts of the central nervous system, fail to complete asymmetric cell division and are arrested in prometaphase-like state with highly condensed chromosomes. Mitotic spindles are unfocused and disorganized and cells exhibit aberrant centrosome numbers. Intriguingly, while all other structures are properly developed, asp mutant larvae display reduced brain size, with extensive lost of cells in the optic lobe and in the eye imaginal disc. Likewise, despite reduced head size and severely impaired eye morphology, asp pharate adults appear morphologically
normal. Altogether, our results suggest that Asp is a neurogenic gene with a major role in the regulation of neural stem cell proliferation and differentiation during brain development.

399/B346
**Electrical Control of Cell Polarization in the Fission Yeast Schizosaccharomyces pombe.**
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Endogenous electric signals surround tissues and cells in all organisms and have been proposed to participate in directing cell polarity in processes such as development, wound healing and host invasion. The application of exogenous electric fields (EF) can direct cell polarization in almost all cell types tested, ranging from bacteria to neutrophils, yet the mechanism for such effects remain a mystery. Here, we introduce the fission yeast Schizosaccharomyces pombe as a model system to elucidate the mechanisms underlying this process. In the rod-shaped fission yeast cells, we show that an exogenous EF reorients cell growth in a direction orthogonal to the field, producing cells with a bent morphology. A candidate genetic screen identifies conserved factors involved in this process: an integral membrane proton ATPase pma1p, the small GTPase cdc42p, and the formin for3p. Interestingly, mutants in these genes still respond to the EF but orient in the wrong direction, towards the anode. We propose that the EF reorients cell polarization by locally modifying a cortical pH gradient set by pma1p, that participate in the regulation of cdc42p and for3p to direct actin assembly and cell growth. Thus, these studies begin to elucidate some of the first molecular insights for mechanisms in the electrical dimension of cell polarization.

400/B347
**Genetic Control of Cell Width in Fission Yeast.**
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Proper cell shape is essential to cell function, but the genetic control of the dimensions of a growth zone has been difficult to understand. The fission yeast Schizosaccharomyces pombe is a rod shaped cell that grows by linear extension at the cell tips, with nearly constant width throughout the cell cycle. This simple geometry makes it an ideal system to study the control of growth zone dimensions. We conducted a visual screen of morphology mutants, screening nearly 3000 viable, haploid fission yeast strains, each carrying a deletion of a single gene. We identified 11 genes that when deleted produce cells whose diameter is at least 10% greater than wild type, though the overall cell volume is unchanged. Six of these wide mutants are involved in the regulation of the small GTPase Cdc42, which is a conserved regulator of polarized growth throughout eukaryotes. To distinguish between defects in the maintenance of cell diameter and defects in cell size establishment we tested the ability of these mutants to repolarize after cell wall removal, which creates a completely round cell. All the wide mutants tested repolarized after cell wall removal, forming a new growth zone with a larger diameter. Genetic analyses showed that at least two different pathways are involved in specifying cell width. When a Cdc42 GAP and GEF were deleted, the effects on cell morphology were additive. We then showed that the GFP-tagged GEF was found at the growing cell tips, and the GAP at the cell sides. By expressing the GFP-tagged proteins in deletions we found that the GEF and GAP localized independently of one another. We hypothesize that these regulators may be acting to set up a zone of active growth at the cell tip and an area where growth is inhibited on the cell sides. This hypothesis will be tested by ectopic targeting of these regulatory proteins to cell sides and cell ends.

401/B348
**The PAM-1 Aminopeptidase Regulates Centrosome Dynamics to Ensure Anterior-Posterior Polarity Establishment in the One-Cell C. elegans Embryo.**
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In the one-cell *Caenorhabditis elegans* embryo, the anterior-posterior (A-P) axis is established when the sperm donated centrosome contacts the posterior cortex. While this contact appears to be essential for axis polarization (1), little is known about the mechanisms governing centrosome
positioning during this process. In a screen for mutants with defects in A-P axis establishment, we identified mutations in \textit{pam-1}, which encodes a puromycin sensitive aminopeptidase. These aminopeptidases have been identified across a large range of species, but little is known of their \textit{In Vivo} functions (2). Previously we showed that \textit{pam-1} mutants fail to polarize the A-P axis and have centrosome positioning defects (3). Here we tested the hypothesis that PAM-1’s role in polarity establishment is to ensure centrosome contact with the posterior cortex. To test this, we inactivated the microtubule motor dynein, DHC-1, and its regulator LIS-1 in an attempt to prevent centrosome movement from the cortex and restore anterior-posterior polarity. This was indeed what we observed. Centrosomes remained anchored to the posterior cortex in \textit{pam-1; dhc-1(RNAi)} and \textit{pam-1; lis-1(RNAi)} embryos. In wild-type embryos, pseudocleavage, posterior localization of P granules, and asymmetric cortical localization of the PAR proteins are signs of proper A-P polarity. These signs of polarity are absent in \textit{pam-1} mutants; but, when combined with \textit{dhc-1(RNAi)} or \textit{lis-1(RNAi)}, we observed normal pseudocleavage and localization of the P granules and PAR proteins. We conclude that DHC-1 and LIS-1 are required for the abnormal centrosome movements in \textit{pam-1} embryos. Additionally, we show that PAM-1’s role in axis polarization is to prevent premature movement of the centrosome from the posterior cortex, ensuring proper axis establishment in the embryo. Thus we have determined a key regulator of centrosome positioning in the one-cell \textit{C. elegans} embryo, that is necessary for polarity establishment. (1) Cowan and Hyman (2004) Nature 431, 92-96. (2) Brooks et al. (2003) J. Biol. Chem. 278, 42795-42801. (3) Lyczak et al. (2006) Development 133, 4281-4292.

**402/B349**

\textbf{Testing the Role of a Putative Arf GTPase-Activating Protein dASAP in Epithelial Cell Polarity in Drosophila.}

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Epithelial cells are polarized with different proteins positioned at their apical and basolateral domains. This is critical for epithelial development and physiology. Among apically localized proteins, Baz/Par3 protein is a key polarity regulator. However, how Baz interacts with other proteins to modulate cell polarity is largely elusive. Therefore, we performed a genetic modifier screen to identify novel Baz interacting genes. Consequently, we found a mutation in \textit{dASAP} (\textit{Drosophila ArfGAP with SH3 domain, Ankyrin Repeat and PH domain}) encoding a putative Arf GTPase-activating protein significantly enhanced the baz zygotic mutant cuticle phenotype. By live imaging of \textit{Baz::GFP} Trap line in the homozygous \textit{dASAP} mutant background, we found Baz::GFP levels were substantially reduced at dorsal closure of embryogenesis. Mammalian ASAP1 is an important regulator of Arf GTPase family which are involved in vesicle trafficking and several actin-based cellular structures. We started to analyze the subcellular localization of dASAP during embryogenesis by live imaging of GFP-tagged dASAP. at cellularization, dASAP appeared to be localized at microvilli and furrow canal of forming epithelial cells. from germband extension to dorsal closure, cortical dASAP became enriched apically, localizing to the apical circumference and the apical surface of epidermal cells and amnioserosal cells. We also documented the movement of dASAP-positive vesicle-like structures in epidermal cells. To see if dASAP colocalizes with polarity and cytoskeleton proteins, we immunostained GFP-tagged dASAP embryos at dorsal closure stage for Baz, Arm, aPKC, Crumbs, actin and tubulin. dASAP generally overlapped with all four apical proteins but local mutual exclusion was evident. Notably, dASAP had relatively high colocalization with actin at both the apical circumference and the apical surface. There were minimal colocalizations with microtubule. Thus, dASAP may regulate actin-based processes at apical domain. Since dASAP localizes to both the apical domain and to intracellular structures, we hypothesize that dASAP may regulate intracellular trafficking to or from the apical domain.

**403/B350**

\textbf{Rab11 Is Important for Chitin Deposition during Drosophila Sensory Bristle Development.}

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Sensory bristles of *Drosophila melanogaster* are polarized single cell extensions whose shafts extend between 70µM (microchaetes) and 500µM (macrochaetes). Their polarized structure makes them useful model systems to study the role of cytoskeleton in cellular morphogenesis. Previous work to understand bristle growth has focused on analyzing how actin filaments are assembled and positioned to mediate shaft elongation. Little is known about the mechanisms that mediate membrane and protein transport to bring about their growth. Our studies identify an important role for dRab11 a small GTPase in bristle development. Knocking down Rab11 function using Rab11dsRNA results in short knob like structures. Mutants with reduced Rab11 function display short bristles with deformed tips. Rab11-GFP preferentially accumulates to the tip of growing bristles. This pattern is altered more in bristles with disrupted microtubules than in those with disrupted actin filaments suggesting an essential role for microtubules in the polarized transport of Rab11. Live imaging studies show that Rab11 knockdown bristles elongate, but at a slower rate than wild types. At a late stage in elongation, bristles start to bulge at various positions along the shaft. These deformities become severe over time and the bristle eventually collapses. This collapse is the cause of small bristle stubs seen in the adult. Bristles with disrupted Rab11 function show dramatic differences in their arrangement of chitin, a key component of the adult exoskeleton. Chitin formation is delayed in these bristles and chitin bundles fail to show the distinct arrangement seen in normal bristles. Our results indicate that Rab11 plays a key role in maintaining bristle stability by mediating formation and/or organization of the chitin exoskeleton.

**404/B351**

Endocytosis Is Required for Apical Constriction during Xenopus Gastrulation.

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Apical constriction is a type of cell shape change that drives epithelial invagination, a process key to morphogenetic events such as gastrulation, neurulation, and organogenesis. We have chosen bottle cells of the Xenopus gastrula, which bend the tissue at the blastopore lip, to study apical constriction. Previously, we showed that actomyosin contractility drives apical constriction in bottle cells; however, it remained unclear whether it does so alone or in concert with other processes. As apical constriction involves rapid membrane remodeling, we hypothesized that endocytosis may be required for apical constriction. We found endosomes exclusively in bottle cells, where some are transcytosed from the apical to the basolateral membrane. Moreover, disrupting endocytosis with dominant negative versions of Dynamin and Rab5 resulted in defective apical constriction. As inhibition of endocytosis did not completely prevent apical constriction, we hypothesized that endocytosis acts as a clearing mechanism during constriction: as the membrane constricts into microvilli, excess membrane is removed via endocytosis. Indeed, inhibition of endocytosis led to a significant and specific decrease in the constriction rate late in the process. Our results indicate that endocytosis is required for apical constriction and acts downstream of actomyosin contractility to decrease apical surface area. We propose that active membrane remodeling during apical constriction could be a general mechanism used by embryos to achieve efficient invagination during morphogenesis.

**405/B352**

The Short Isoform of PDGF-A Is an Instructive Signal Regulating Directional Intercellular Migration of Pre-Chordal Mesodermal Cells during Gastrulation in *Xenopus*.

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The movements of leading edge mesendoderm during gastrulation in *Xenopus* have been well characterized, however the movements of the pre-chordal mesoderm have not been investigated in detail. It is thought that cells within the deep layers of the pre-chordal mesoderm undergo intercellular migration, a poorly understood process in which cells use the surfaces of other cells as a substrate for migration. Using scanning electron microscopy, we show that deep pre-chordal mesodermal cells are unipolar and are oriented toward the ectodermal blastocoel roof (BCR). We also show through timelapse recordings of mesoderm-BCR explant combinations that pre-chordal
mesodermal cells migrate directionally toward the BCR in an intercellular fashion. Platelet derived growth factor (PDGF) ligands and receptors are known for their roles in mitogenesis, angiogenesis, cell death regulation, and directional cell migration. We show that disruption of PDGF-A function by microinjection of morpholino oligonucleotides or dominant negative constructs inhibits directional migration of deep pre-chordal mesodermal cells. This effect is reversible by expression of the wild-type short isoform of PDGF-A in the BCR. We also show, using mesoderm-endoderm explant combinations, that PDGF-A is sufficient to regulate directional intercellular migration of these cells. Altogether, our data suggest an instructive role for the short isoform of PDGF-A as a chemoattractant in the directional migration of pre-chordal mesodermal cells. This research contributes to a further understanding of the process of mesoderm migration during gastrulation in Xenopus as well as introducing an In Vivo model to further the understanding of intercellular migration.

**406/B353**

**Polarity of Microtubule Cytoskeleton during Zebrafish Gastrulation.**

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Vertebrate gastrulation includes convergence and extension (CE) movements that narrow and elongate the embryonic body. Multiple cell behaviors underlie these movements; some require Wnt/Planar Cell Polarity (Wnt/PCP) signaling. Cellular phenotypes dependent on Wnt/PCP signaling during gastrulation are typically interpreted as aspects of cell polarity; e.g. cells elongate, align and migrate, divide or intercalate perpendicular to the body axis. To investigate mechanisms of Wnt/PCP dependent polarity we are examining the microtubule cytoskeleton during zebrafish gastrulation. An early step in migration of cultured cells is often the alignment of the nucleus and microtubule organizing center (MTOC) toward the direction of migration. Recent work shows Wnt5a, and its effector Dishevelled, are required for MTOC reorientation in cultured cells (Schlessinger et al, 2007, Nomachi et al, 2009). Here we show the microtubule cytoskeleton is aligned in gastrula cells and this is dependent on some components of Wnt/PCP signaling. To assess polarity in live zebrafish embryos at late gastrulation, we collected confocal images of MTOC and nucleus labeled with Xcentrin-GFP, cell membrane with RFP. Cells were scored for orientation of the MTOC relative to the nucleus and the dorsal direction of migration. We found that cells of both lateral ectoderm and mesoderm have a polarized arrangement of the nucleus-MTOC-leading edge during late gastrulation. The orientation of the MTOC is randomized in embryos in which some Wnt/PCP signaling components are disrupted or at a younger stage when lateral mesoderm migration does not require Wnt/PCP. These results indicate Wnt/PCP signaling controls MTOC polarity during gastrulation movements. To understand function of the microtubule cytoskeleton during convergence and extension, we are disrupting microtubule dynamics at late gastrula stages, and examining tissue morphogenesis, and cell properties associated with Wnt/PCP. Notably Prickle and Dishevelled are asymmetrically localized by late gastrulation or early segmentation. We find that the maintenance of Prickle localization does not require dynamic microtubules.

**407/B354**

**Mirror Symmetry Relationship between Sister Cells.**

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Cell shape determination is a dynamical process modulated by input from genetic and signaling pathways. In order to understand cell morphology as a dynamical system it is critical to determine the degree of spatial inheritance, that is, the degree of influence of spatial organization within a mother cell on the organization of her daughter cells. Visual comparison of symmetry relations between daughters was used by Albrecht-Buehler to probe spatial inheritance in a series of papers published in the 1970's. His results, that sister cells are sometimes mirror images of each other, were interpreted as reflecting spatial inheritance during cell division. We have reinvestigated these claims using quantitative image analysis and several different shape-comparison algorithms to test the symmetry relations between sister cells. Applying these methods to fixed and live RPE-1 and NIH 3T3 cells, we obtained the following results: (A) sister cells are quantitatively more similar in shape than pairs of unrelated cells, (B) When sister cells show a significant degree of shape similarity, they tend to be related by mirror symmetry, (C) the shape similarity between sister cells is highest soon after division and decays on a time scale of several hours, (D) the set-theoretic union of the two sister cell shapes is related to the shape of the mother with a degree of similarity that decays as a function of time before and after division. We have also developed methodologies to compare similarity of internal actin stress fiber organization between sisters and to compare the migration trajectories of sister cells as they move away from the site of division.

PTK7 Proteolysis by MT1-MMP Regulates Embryogenesis and Cancer.
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PTK7 (colon carcinoma kinase-4, CCK-4) and MT1-MMP (membrane type-1 matrix metalloproteinase, MMP-14) are membrane proteins that are essential for non-canonical Wnt signaling, planar cell polarity (PCP) and convergent extension (CE) during gastrulation. Increased expression of both PTK7 and MT1-MMP are associated with the progression of multiple types of cancers. Our proteomics studies on normal and cancer cells identified PTK7 as a cleavage target of MT1-MMP. We determined that MT1-MMP cleaves the PTK7 ectodomain at the PKP621-LI site and generates the PTK7 soluble form. It was shown by others that soluble PTK7 antagonizes the function of the membrane PTK7. To determine the significance of the MT1-MMP-dependent generation of soluble PTK7 on the organism level, we used the zebrafish vertebrate model system. Injection of the mRNA encoding the human soluble PTK7 as well as the transcriptional silencing of MT1-MMP and PTK7 with specific morpholino antisense oligonucleotides, all resulted in similar defects normally associated with aberrant PCP and CE. Our results suggest that the soluble human PTK7 can antagonize the function of the zebrafish membrane PTK7, and that both the membrane PTK7 expression and MT1-MMP activity are essential for the normal embryonic development. These findings suggest that the MT1-MMP proteolysis of PTK7 is tightly controlled during embryogenesis and both the excess and the lack of soluble PTK7 adversely affect embryogenesis. Further, our studies in cancer cells revealed that the levels of the membrane PTK7, which co-localizes with MT1-MMP at the cell-cell junctions, inversely correlated with cell invasion and MT1-MMP activity. In addition, we find that the levels of PTK7 in breast tumor tissue were lower and inversely correlated with the stage of tumor progression. We hypothesize that upregulation of both PTK7 and MT1-MMP would lead to increased soluble PTK7 and subsequent defects in non-canonical WNT signaling and planar cell polarity, which in turn would promote malignant transformation.

An Insulin-Mediated Stem Cell Response Controls Adaptive Intestinal Morphogenesis in the Adult Drosophila Midgut.
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In adult animals, differentiated tissues must continually adapt to changing environmental and physiological demands. Remodeling of the vertebrate intestinal lining in response to fluctuating dietary loads is a prime example of such adaptation. However, the cellular mechanisms of such post-developmental morphogenesis are poorly understood. Using a new model of adult organ remodeling, we show that intestinal stem cells in the mature Drosophila midgut interpret an instructive nutrient signal to direct tissue growth toward the functionally appropriate state. Food ingestion causes an increase in intestinal size and cell number. This adaptive growth occurs via insulin-activated stem cell proliferation. Synthesis of insulin matches the kinetics of stem cell proliferation in fed and fasted conditions. Insulin receptor activation in stem cells stimulates both symmetric and asymmetric divisions, thereby elaborating the intestinal epithelium when food is abundant. Altogether, these data suggest that mature organs can respond to external cues for adaptive change by leveraging programs of stem cell renewal. We postulate that in the gut, the nutrient-triggered stem cell response enables organ morphogenesis to be tuned to functional need.

410/B357
The Role of Insulin Signaling and Nutrition in the Regulation of Drosophila Male Germline Stem Cells.
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A steady supply of differentiated cells from adult stem cells is critical to maintain tissue homeostasis. Defective control of stem cell number either results in loss of stem cells leading to tissue degeneration, or excess proliferation of stem cells leading to tumorigenesis. The Drosophila male germline stem cell (GSC) provides an excellent model system to study stem cell behavior in the context of the stem cell niche. Their niche is located at the tip of the testis. GSCs attach to a cluster of somatic cells called the hub, which provides an essential ligand (Upd) for stem cell identity, and divide asymmetrically by orienting their mitotic spindle perpendicularly to the hub. Stereotypical positioning of centrosomes with respect to the hub sets up this spindle orientation; one centrosome (mother centrosome) always locates close to the hub-GSC junction, while the other centrosome (daughter centrosome) migrates to the opposite side of the GSC. Recently, we have shown that GSCs with misoriented centrosomes, where neither of the two centrosomes is juxtaposed to the hub-GSC junction, undergo cell cycle arrest until their centrosomes reorient, suggesting a novel cell cycle checkpoint to monitor correct centrosome positioning. Here we show that nutrition, through the insulin pathway, may have an effect on centrosome orientation in GSCs. Flies grown in poor nutrient conditions have significantly higher frequency of GSCs with misoriented centrosomes. This response appears to be mediated via the insulin signaling pathway, since expression of constitutively active insulin receptor in GSCs restores centrosome orientation even when cultured in poor nutrient conditions. We propose that the nutrients’ condition modulates GSC division rates by controlling centrosome orientation, which can regulate cell cycle progression via the centrosome orientation checkpoint.

411/B358
Reserve Stem Cells of Asexually Reproducing Organisms.
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Asexual reproduction is a natural cloning of oozooid, with development of many genetically and morphologically identical blastozooids. In asexually reproducing animals, the lineage of reserve stem cells ensures both sexual and asexual reproduction, being predecessors of germline and all somatic cells. We studied morphological and functional organization of reserve SCs in five animal types: Porifera (Oscarella malakhovi), Cnidaria (Obelia longissima), Platyhelminthes (Girardia tigrina), Arthropoda (Pelagogasterella gracilis, Polycascus polygenea), Chordata (Botryllus tuberatus) and mouse ESC in vitro. Asexual reproduction without separation of blastozooids in
some rhizocephalans results in the emergence of colonial organization that is unique among crustaceans, all arthropods, and all Ecdysozoa. Blastogenesis and coloniality in the rhizocephalans involve a radical, evolutionary secondary rearrangement of the ancestral reproductive biology due to the transition to parasitic life. We found germinal bodies in all or most blastomeres in cleaving embryos of P. polygenea. Each body selectively expresses mRNA transcript of vasa-like gene. This data indicates a saltatory evolution from determined mosaic cleavage with preformation to regulative development with epigenesis. The morpho-functional organization of stem and gonial cells in the studied species shares common properties: A high nuclear/cytoplasmic ratio, a large nucleus with diffuse chromatin and a large nucleolus, basophilic cytoplasm including specific perinuclear germinal granules, the positive reactions revealing proliferating cell nuclear antigen and alkaline phosphatase activity. We observed electron-dense granular structures in mESCs similar to germinal granules in metazoan oogenic cells. Similar structures were found in reproductive cells of lower and higher plants. This similarity indicates a very conservative pattern of reproductive cells in Metazoa and Metaphyta. Thus, our data indicates evolutionary conservatism and common morpho-functional organization of germ cells from all studied multi-cellular animals and unlimited morphogenetic potential of reserve stem cell capable of both gametogenesis (and embryogenesis) and asexual reproduction (blastogenesis).

412/B359
Notch Signaling Is Activated during Regeneration of the Airway Epithelium.
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Objective: Notch signaling plays an important role in establishing the balance between ciliated and secretory cell fates in the developing airways. However little is known whether this mechanism is present during regeneration of the airway epithelium in adult life. The goal of the study is to identify the Notch pathway components in adult airways undergoing regeneration after naphthalene injury. Methods: Adult CD1 mice were injected intraperitoneally with naphthalene and airway epithelium desquamation and regeneration were investigated, including changes in morphology, expression of differentiation markers and Notch components. We used in situ hybridization and immunohistochemistry on lung tissue sections. Results: Overall expression of Notch components/expression was nearly undetectable in adult lungs. at 52 hr of naphthalene injury, when secretory cells were ablated in the airway, the Notch signaling effectors (Hes1, Hey1, Hey2 and HeyL) remained low in the remaining cells. However by 72 hr, when the regeneration process started, Notch1 and the ligand Jagged1, as well as Hes1 and Hey1 were up-regulated in the airway epithelium with distinct patterns. Expression of Hey2 and HeyL remained low at all time points. Interestingly, up-regulation of Notch1 and Hes1 correlated with increased expression of Scgb3a2 (secretoglobin, family 3A, member 2), an early secretory cell marker. Additionally, increased Notch1 and Hes1 expression was observed almost exclusively in cells negative for Foxj1 (forkhead box j1), an early ciliated cell marker. These suggest that activation of Notch signaling, likely by Notch1, may favor the acquisition of the secretory cell phenotype during the repopulation of the airway epithelium after injury. Conclusions: Thus Notch signal may be crucial for the balance of differentiated cell fates during airway regeneration, as it is in development.

413/B360
Inscuteable Drives Asymmetric Cell Divisions in the Mouse Epidermis.
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Asymmetric cell divisions (ACD) are used throughout development to promote different fates between two daughter cells. In the mouse epidermis ACD within the proliferative basal layer promote stratification and differentiation. Unlike invertebrate model systems for ACD, which undergo obligate asymmetric divisions, basal cells have the capacity to undergo both asymmetric and symmetric divisions. Thus, a basal cell must be able to direct the proper orientation of its mitotic spindle to achieve the correct type of division. We provide evidence on how and when this decision is made. To this end, we used two transgenic mouse lines that labeled either the centrosomes or spindle poles. By quantifying their localization throughout the cell cycle, we have determined that basal cells do not establish their axis of division until metaphase via spindle
rotation. This phenomenon has been verified in cultured keratinocytes expressing centrin-GFP. This differs from invertebrates that establish their spindle orientation prior to nuclear envelope breakdown via centrosome migration. Thus basal cells may have the ability to choose between dividing symmetrically or asymmetrically even after committing to mitosis. ACD's require the formation of an apical protein complex. Inscuteable is a component of the ACD machinery that is necessary and sufficient to drive some ACDs in Drosophila. We have generated mice that allow inducible expression of mInscuteable in the epidermis. Upon induction of mInscuteable, the protein localized normally to the apical cortex and increased the occurrence of ACDs. From this we conclude that Inscuteable is sufficient to induce ACD and therefore is likely a key regulatory element in the process of stratification. Further exploring these questions will give us insight into how the spindle orientation machinery functions to achieve proper alignment and how basal cells use asymmetric divisions to properly develop.

414/B361
Homeostasis of Infrequently Dividing Hair Follicles Stem Cells by Symmetric Daughter-Cell-Fate Determination.
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Regenerative tissues contain stem cells that self-renew and differentiate throughout life. In normal vertebrate tissue homeostasis, stem cells are predicted to rarely divide and adopt an asymmetric progeny-cell fate: one daughter remains a rarely dividing stem cell and the other becomes a rapidly dividing short-lived progenitor. In order to test such hypotheses, we employ In Vivo division tracking and stem cell fate lineage tracing, to examine the long-term dynamic behaviour of hair follicle stem cells in mouse skin. We provide strong In Vivo evidence at the single-cell resolution for the infrequent stem cell division model in this vertebrate system. We find that proliferation and differentiation of progenitor cells occur at different times and tissue locations than self-renewal of stem cells. Distinct fates of differentiation or self-renewal are assigned to individual stem cells in a temporal-spatial manner. We propose that large groups of tissue stem cells behave as populations, in which pool maintenance involves symmetric daughter-cell-fate decisions.

415/B362
Regulation of Homeostasis of Hair Follicle Stem Cells by Bmp Signalling.
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During the hair cycle, the behaviour of slow-cycling, hair follicle stem cells (hf SCs) is tightly governed by an intricate balance of signalling pathways which converge to induce bouts of SC quiescence and activation, resulting in new hair formation. We have previously shown that bone morphogenetic protein (BMP) signalling is essential for hair follicle stem cells homeostasis, revealing ablation of the Bmpr1a gene resulted in the precocious activation of quiescent hf SCs and premature expansion of the SC niche (Kobielak et al., 2007). However, precisely how BMP signalling functions in hf SCs at the molecular level still remains to be elucidated. Since CD34 the only available marker for isolation of hf SCs was lost upon BMPR1A ablation to address this question we employed, a Keratin 15 (K15)-driven model to simultaneously label and specifically target BMPR1A ablation within the hf SCs during the second postnatal telogen. Using this approach we were able to isolate hf SCs marked by eYFP from control and BMPR1A knockout (KO) by fluorescence activated cell sorting (FACS) before morphological changes in hair cycle were indicated. Transcriptional profiling reveals that after ablation of BMP signaling in hf bulge, stem cells show downregulation of approximately 25% of common upregulated hf SCs signature genes (Greco et al., 2009 and Blanpain et al., 2004). at the same time they acquire molecular characteristic of hair germ as our data show that 30% of genes overlapped with previously characterized hair germ signature (Greco et al., 2009) Our findings suggest a model where balancing BMP signalling in hair follicle stem cells is important to maintain their homeostasis and
its inhibition could be important to switch them from their quiescence to activation towards hair germ.

416/B363

FGF2 Maintains the Proliferation of Neural Precursors by Actively Suppressing the Cki p27Kip1 through Regulation of Cks1b Transcription.

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Identifying the mechanisms that regulate the proliferation and differentiation of neural precursor cells (NPCs) is important for understanding CNS development in vertebrates. We hypothesize that during embryogenesis, NPCs in the ventricular zone (VZ) maintain their proliferation in response to growth factors by repressing the cell cycle inhibitor p27Kip1, in part, through the up-regulation of Cks1b and activation of the degradation complex SCFSkp2. We report that while p27Kip1 mRNA is detected in the VZ and in the mantle zone of the spinal cord and cortical plate of the cerebral cortex, p27Kip1 protein is mostly absent from the VZ throughout the neuraxis. We show that NPCs harvested from E14 rat cerebral cortex in the presence of FGF2 express high protein levels of Cks1b and undetectable levels of p27Kip1, while removal of FGF2 causes a sharp decrease in Cks1b and corresponding increase in p27Kip1. Furthermore, our RNA expression data shows that Cks1b mRNA is reduced in non-dividing NPCs but is present in proliferating progenitors, suggesting that Cks1b is being regulated at the transcriptional level. Analysis of the putative promoter of Cks1b revealed numerous conserved transcription factor consensus sites, including E2F and cell cycle-dependent element (CDE) cell cycle genes homology region (CHR) consensus sites. We used chromatin immunoprecipitation to identify which E2Fs occupy the Cks1b promoter under different conditions of mitogen stimulation. Our results show that the E2F4/p130 complex occupies the Cks1b promoter in quiescent NPCs while E2F1 binds the Cks1b promoter in proliferating NPCs. In a luciferase reporter assay, mutation of either the E2F or CDE/CHR consensus sites de-represses Cks1b promoter activity in NPCs in G0/G1, while mutation of both sites together delays induction of promoter activity. Finally, we used a gain-of-function approach using in ovo electroporation to address whether or not p27Kip1 has a moonlighting role in neuronal differentiation. We find that ectopic expression of p27Kip1 reduces the number of proliferating cells in the early spinal cord, diminishing its overall size and shape, but is insufficient to induce neuronal differentiation in spinal cord neural precursors.

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Lens Development in the Zebra Fish Resembles the Mammal.

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OBJECTIVE: The progressive stages of development from lens placode to optical element was studied in real time to test the hypothesis that the primary fibers in the embryonic zebra fish lens originate in the central placode and the epithelial cells originate in the periphery of the placode. METHODS: 2-photon live-embryo imaging was conducted on Q01zebrafish that express cyan fluorescent protein fused to a membrane-targeted sequence of zebra fish Gap43 (mCFP), driven by an EF1α promoter and a hexamer of the DF4 pax6 enhancer element. Each differentiating cell was outlined. Fertilized Q01 zebra fish eggs were injected at the 1-cell stage with a combination of two plasmids, EF1α::Gal4VP16 containing a Pax6 enhancer and UAS::td-tomato, for mosaic expression of fluorescent tomato red protein. RESULTS: Cells in the peripheral lens placode migrated to the anterior lens mass and differentiated into the lens epithelium. Cells in the central lens placode migrated to the posterior lens mass and differentiated into primary fiber cells. Anterior and posterior polarization of the zebra fish lens cell mass resembled the anterior and posterior polarization of the mammalian lens vesicle. TUNEL labeling demonstrated that apoptosis was not a primary mechanism for separation of the lens from the surface ectoderm and future cornea. BrdU incorporation revealed that proliferation was restricted to a lateral zone in the
lens epithelium similar to the mammal. Fiber cell differentiation began prior to separation of the lens mass from the surface ectoderm, as evidenced by cell elongation, exit from the cell cycle, and expression of Z1-1, a lens fiber cell marker. Protein analysis of zebrafish lens homogenates at selected ages from 4 days to 6 months post fertilization determined that the variation in protein content with age resembled that of mammals. CONCLUSION The results confirmed the hypothesis that a symmetric, refractile, transparent zebrafish lens was generated from a sheet of undifferentiated, embryonic, cranial epithelium using biological mechanisms that are similar to mammalian lens development. Supported by EY04542 from the NEI. Dedicated to the memory of Prof. DW Fawcett.

418/B365
Sox2 and the Xenopus Homologue of Oct4 Maintain Neural Progenitors in Xenopus laevis.
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Since Sox1, Sox2 and Sox3, the three members of the SoxB1 subgroup, have similar expression patterns, protein structures and functions, it has been suggested that they have redundant roles during neural development. Here we show that Sox1, Sox2, Sox3, and Oct91, the Xenopus homologue of Oct4, have overlapping and distinct roles in inducing neural progenitor identity. In naïve ectoderm using ectodermal explant assays, Sox1, Sox2, Sox3 induce a unique array neural genes markers but only Sox1 is sufficient for neuronal differentiation. Sox2 and Sox3 function independently of each other, yet Sox1 induces expression of both Sox2 and Sox3. While Sox2 induces limited neural progenitor markers, Sox3 induces a broad profile of neural progenitor and pro-neural markers and this may be due to its unique induction of FGF8 expression. Sox3-VP16, the dominant activator form of Sox3, does induce neuronal markers and Sox2 expression, which indicates that Sox3 requires the presence of a co-factor for neuronal differentiation. Only a few co-factors of the Sox proteins have been identified and therefore their function in combination with a partner has not been tested in early development of the nervous system. In naïve ectoderm, Oct91 induce sox1-3 expression and induces neuronal differentiation. Overexpression of both Sox2 and Oct91 maintained neural progenitor markers while also inhibiting neuronal differentiation. However, the addition of Sox1 or Sox3 abolishes the ability of Oct91 to induce any neural markers even though epidermis formation was still repressed. Finally, transgenic embryos were generated using a bicistronic expression vector to determine if transient overexpression of the SoxB1s delays neuronal differentiation due to loss of the mRNA over time. Constitutively-active expression of Sox1 or Sox2 transgenic embryos represses neuronal differentiation indefinitely while also increasing cell proliferation. Constitutively-active Sox3 leads to cell death.

419/B366
TXNIP Regulates Hematopoietic Stem Cell Quiescence and Mobilization.
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Hematopoietic stem cells (HSCs) are maintained in a quiescent state in bone marrow (BM) niches by intrinsic and extrinsic signals. However, the mechanism that coordinates the cell cycle regulation of HSCs and the interactions between HSCs and the niche remains unclear. Thioredoxin-interacting protein (TXNIP) is known as a critical regulator of a broad range of cellular functions, including maturation of natural killer cells, immune regulation, glucose and lipid metabolism, and regulation of the life span, aging, renal function, homeostasis, and hematopoiesis. Here, we investigated the role of TXNIP in hematopoietic stem cell quiescence and mobilization using Txnip⁻/⁻ mice. The expression of TXNIP measured by quantitative real-time RT-PCR was decreased during HSC activation. Flow cytometry analysis and reconstitution assay showed that the long-term reconstituting HSC population was decreased and exhausted, and its capacity to repopulate was rapidly lost in Txnip⁻/⁻ mice. These effects are associated with hyperactive Wnt signaling, an active cell cycle, and reduced p21 expression under conditions of stress. TXNIP deficiency reduced the CXCL12- and osteopontin-mediated interaction between HSCs and the BM, and impaired homing and retention in the osteoblastic niche, resulting in...
mobilized HSCs. Therefore, we propose that TXNIP is essential for maintaining HSC quiescence and the interaction between HSCs and the BM niche.

**420/B367**

**Fever Like Temperature Increases and Accelerates Erythroid Differentiation through Normal Regulatory Circuitry.**

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The differentiation of hematopoietic stem cells (HSC) along the erythroid lineage is regulated by the cytokine erythropoietin (EPO). Central within the erythroid differentiation program is the transcription factor GATA-1 which regulates a wide array of genes required for the development of red cells. The heat shock factor protein HSP70 was recently shown to have important role in erythropoiesis through the regulation of GATA-1. This led us to test whether mild hyperthermia (MH) such a fever (39°C), could impact the differentiation of HSCs into erythrocytes. Our hypothesis is that MH would promote erythroid differentiation due to increased GATA-1 activity due to increased HSP70 expression. Cord blood CD34+ cells were grown at 37 or 39°C in medium supplemented with EPO and SCF. Cultures maintained at 39°C experienced a profound acceleration in erythroid differentiation as evident by accelerated appearance of glycophorin a (GPA)+CD71+ cells (66±3 vs 43±14 at day 5, at 39 and 37°C, n=2), and increased expansion of GPA+ cells (72±11 vs 36±8 x10^3 GPA+ cells at day 14, at 39 and 37°C, respectively n=3). Next, we set out to identify the molecular mechanisms responsible for this. First, we investigated the impact of MH on signal transduction molecules known to regulate erythroid differentiation through the EPO receptor (anti-phosphos antibodies and intracellular cytometry); these analyses revealed that MH led to the activation of STAT5 (2.6±1.9 fold), Erk1/2 (1.5±0.26 fold) and AKT (1.5±0.1) (n=3). Activation of these molecules by MH was not strictly dependent on EPO and addition of inhibitors to these molecules severely impaired (35-100%) the effect of MH suggesting that the effects of MH were mediated in part through these effectors. Secondly, we found that MH led to a strong increase in the expression of HSP70 (2-5X), and increased phosphorylation of GATA-1 at serine 310. Consistent with this, a rapid increased in GATA-1 transcriptional activity was observed in luciferase assay (1.3±0.3 fold,n=3). These results demonstrate that fever like temperature can promote the differentiation of progenitor cells along the erythroid pathway through normal regulatory circuitry that are sensitive to heat shock responses.

**421/B368**

**Hematopoietic Stem and Progenitor Cell Division and Dynamics Modulated by Non-Muscle Myosin II Inhibition.**

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Investigation of mechanisms behind hematopoietic stem and progenitor cell (HSPC) division is of clinical importance, including identification of optimal ex vivo culture conditions for HSPC expansion. Contributions of cytoskeletal machinery to HSPC division and population dynamics remain largely uninvestigated. One important cytoskeletal component of cell division is non-muscle Myosin II (NMM II) which mediates furrow ingression during cytokinesis. In the present study, contributions of NMM II to HSPC division were evaluated under ex vivo culture conditions that were previously used to expand human CD34+ cell number in clinical studies. This is followed by pharmacological modulation of NMM II ATPase activity by (±)-blebbistatin, a selective NMM II inhibitor, combined with subpopulation analysis using flow cytometry with defined surface markers for HSPCs. Cell number of the primitive HSPC subpopulation, CD34+CD90+ (~20% of total at day 0 culture) was maintained for 4 days and then rapidly declined to nearly 0% on day 7. In contrast, cell numbers of less primitive subpopulations, CD34+CD90- and CD34-CD90-, were slowly increased over 4 days. Afterwards, the number of CD34+CD90- cells remained relatively
constant, while that of CD34^+CD90^- was dramatically increased on day 7. Cells were treated with different doses of blebbistatin on day 4, when active cell proliferation was taking place (~3 fold increase from day 0). High concentration (>25uM) of blebbistatin led to progressive cell death, while 12.5uM of the drug reduced the total viable cell number (1.5~2 fold compared to untreated), but overall increase in cell number (2 fold increase from 0hr treatment) in 60hr treatment. An initial experiment also raises a possibility that blebbistatin may lead to alterations in HSC population dynamics - 12.5uM blebbistatin treatment for 60hr led to 2 fold increase in CD34^+CD90^- fraction (42.3% vs 18.2% of total) compared to untreated controls. In conclusion, the present study raises a possibility that NMM II may contribute to HSC cell division and population dynamics. Dose-dependent effect of blebbistatin on HSC subpopulation, cell division and cycle kinetics is currently being investigated.

422/B369
Hematopoietic Progenitor Cell Communication with the Niche Microenvironment Is Regulated by a Specialized Plasma Membrane Domain.
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The spatial organization and dynamic movement of proteins and lipids in cell membranes is essential for regulating cell-signaling and cell communication events. within the bone marrow niche, cell communication between hematopoietic progenitor cells (HPCs) and niche cells provides critical cues for their proliferation and survival. Work from our laboratory identified a specialized plasma membrane domain on HPCs that serves as the contact site with osteoblasts, members of the marrow niche. Characterization of this domain using primary CD34+ cells and the KG1a cell line identified the enrichment of specific tetraspanin family proteins including CD81 and CD63, which are known to organize the plasma membrane into domains important for signaling. Using live cell imaging techniques, we observed the intercellular transfer of proteins and lipids from the HPC specialized domain to osteoblasts. Transferred material was internalized by the osteoblasts into SARA-positive, signaling endosomes, which resulted in the downregulation of Smad signaling and an increase in stromal-derived factor-1 production, a chemokine responsible for HPC homing. Next, we evaluated the functional significance of the specialized membrane domains for In Vivo homing of HPCs to the bone marrow microenvironment. Disruption of HPC membrane domains by cholesterol sequestration had no effect on cell viability, proliferation or colony forming capacity in vitro. However, we observed an over three-fold decrease in homing of HPCs with disrupted membrane domains to the bone marrow of NOD/SCID IL2r ^-/- mice as compared to mock-treated HPCs. Given the known homing/engraftment defect of actively cycling HPCs, we went on to compare membrane domains on quiescent and cycling CD34+ cells. When HPCs were stimulated with cytokines to enter the cell cycle, the polarized morphology and the tetraspanin-enriched domain became undetectable. These findings suggest the importance of tetraspanin-enriched HPC membrane domains for cell communication and homing within the marrow niche. Furthermore, the turnover of this tetraspanin-enriched domain during the cell cycle suggests an importance for this plasma membrane site in maintaining the primitive phenotype of HPCs.

423/B370
MT1-MMP in B Cell Development.
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The development of early stage B cells occurs in bone marrow before they are recruited to peripheral organs during immature B cell stage and then develop into mature B cells. In bone marrow, B cell precursors derive from hematopoietic stem cells (HSCs). They first commit into pro-pre-B cells, and then go through pro-B cell stage, pre-B cell stage and immature B cell stage. It is demonstrated that stromal cells play an important role in organizing the bone marrow niches in which B cells can develop in an ordering way before going into pre-B stage and then leave the stromal cells. During different stages, a serial of different microenvironment factors support the
process. MT1-MMP is a membrane-tethered matrix metalloproteinase which is first found on tumor cell surface. It is critical for bone formation and adipogenesis. Loss of MT1-MMP manifests severe defects in both bone formation and adipogenesis. Here we show that MT1-MMP is expressed in bone marrow derived stromal cells. Because bone marrow stromal cells are important components of bone marrow niches in supporting B cell development, we explored the role of MT1-MMP in bone marrow B cell development. We found that loss of MT1-MMP leads to abnormal reduced B cells population in bone marrow. However, MT1-MMP is not expressed by B cell itself. In addition, reconstitution of MT1-MMP deficient bone marrow cells into lethally irradiated wild type mice can rescue the defect of B cell development, which indicating that the defect is not cell autonomous but comes from a defect of bone marrow niches. Furthermore, co-culture experiment showed that In Vitro cultured wild type bone marrow stromal cells support B cell development better than the stromal cells from MT1-MMP deficient mice. In this way, we demonstrated that MT1-MMP regulates the properties of bone marrow stromal cells and consequently it contributes to the microenvironment in supporting normal B cell development.

424/B371

Epigenetic Regulation of Sox9 during Chondrogenesis.

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Cell-base therapy has been evolved as one of the most popular strategies to treat various bone and cartilage disorders. Comparing with pluripotent stem cells, unipotent patient specific cells with defined differentiation potential might be more applicable in regenerative medicine. Osteo-chondroprogenitor with dual skeletogenic potentials has been considered as the key for bone and cartilage regeneration. However, the underlying mechanism directing the formation and maintenance of osteo-chondroprogenitor remains elusive. Sox9, an SRY related high mobility group family of transcription factor, is a master regulator for chondrogenesis based. In developing mouse embryos, Sox9 expression is initiated in the core of the limb bud mesenchyme at E10.5. Genetic lineage tracing analyses showed that Sox9 expressing cells in the limb bud mesenchyme and its progeny are able to form cartilage and bone, suggesting that they are osteo-chondroprogenitors. These bipotent progenitor cells expressing Sox9 will undergo mesenchymal condensation, proliferation and subsequently differentiation into hypertrophic chondrocytes where Sox9 is downregulated. Understanding how Sox9 expression in the osteo-chondroprogenitor is regulated would provide insight of how the cells are formed and maintained. While the chondrogenic enhancer and upstream regulatory factors directing Sox9 expression remain to be identified, epigenetic has been evolved to be an important element controlling gene activation and silencing. Several In Vitro evidence suggest that Sox9 expression can be subjected to epigenetic regulation. Using bisulfite sequencing, we found that DNA methylation, which appears to be more tissue specific, is rarely detected within the Sox9 proximal promoter region both before and after osteo-chondroprogenitor formation. The data suggests that there could be other epigenetic modifications regulating Sox9 expression at early stages of chondrogenesis. This is the first In Vivo study of DNA methylation profiling on Sox9 in limb bud formation. Understanding the epigenetic profiling of Sox9 would benefit the mechanistic study leading to In Vitro generation of osteo-chondroprogenitors.

425/B372

Identification of the Genes Involved in Signal Transduction during Myogenesis by Using LA-PCs.

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[Objective] Identification of the genes involved in myogenesis can facilitate the development of regenerative medicines. In our previous study, we established left atrium-derived pluripotent cells (LA-PCs). When these cells were cultured in Methocult GF-M5334 (a semi-solid methylcellulose medium) containing cytokines (IL-3, IL-6, and SCF) or grown to high confluence, they
differentiated into functional cardiac myocytes and/or skeletal muscle cells. The objective of this study was to identify the genes involved in myogenesis by using the LA-PCs and performing microarray analysis. We especially focused on the genes involved in signal transduction during myogenesis. [Method] We extracted total RNA from undifferentiated LA-PCs (UND/LA-PC) and fully differentiated LA-PCs (D/LA-PC). Microarray analysis was performed using an Agilent DNA chip containing the rat whole genome. The genes that exhibited more than 2-fold alteration (between UND/LA-PC and D/LA-PC) in expression were selected as candidate genes, and RT-PCR was performed to examine their expression levels. [Results] We observed that the following genes were up-regulated: troponin T1 slow skeletal; myogenin; MEF2C; troponin C. These genes were previously known to be associated with myogenesis. These findings suggested that our technique was efficient in identifying the genes involved in myogenesis. Therefore, using this technique, we further identified the other candidate genes associated with signal transduction. RT-PCR analysis showed that although the noggin gene was expressed in the undifferentiated cells, its expression was down-regulated during the course of myogenesis. In contrast, the expression of IGF-1 and the Wnt inhibitory factor-1(Wif-1) were up-regulated during myogenesis. These findings suggest that the IGF-1 and/or the Wnt signal pathways were involved in myocyte differentiation. [Conclusion] We successfully established the LA-PCs that showed myogenic differentiation. By comparison expression levels of UND/LA-PC with D/LA-PC, we could identify the genes involved in signal transduction during myogenesis.

426/B373

The Role of Homeodomain Factor Barx2 in Muscle Development and Regeneration.

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Adult mammalian muscle regenerates upon injury by activation of undifferentiated myogenic precursor cells called satellite cells, which are normally quiescent and situated between the plasmalemma and the surrounding basal lamina of muscle fibers. Upon activation, satellite cells proliferate and divide asymmetrically, producing one daughter cell that proceeds to myogenic differentiation and another that becomes quiescent and replenishes the satellite cell pool. We are investigating the role of the homeodomain factor Barx2 in skeletal muscle development and regeneration. Barx2 is co-expressed with paired-box transcription factor Pax7 in progenitor cells of fetal and adult muscle and its expression is up-regulated after muscle injury. Mice lacking the Barx2 gene show muscle regeneration defects, reduced muscle growth, and reduced Pax7 expression, suggesting a role for Barx2 in satellite cell survival and/or self-renewal. Cultured Barx2-/- satellite cells are less proliferative and slower to differentiate than Barx2+/- cells, and also show reduced expression of myogenic factor MyoD and myogenin. These findings suggest that defects in muscle regeneration in Barx2 null mice may be caused by reduced activation and/or delayed differentiation of satellite cells. Moreover, interbreeding of Barx2 mutants with muscular dystrophy model mice (mdx) leads to striking dystrophic features. These are characterized by muscle degeneration and the appearance of fibrous infiltrates, mimicking the dystrophic phenotype observed in human patients. Together, our ongoing work suggests that Barx2 is a critical component of the muscle growth and repair machinery in mammals and that it controls myogenic commitment and differentiation.

427/B374

Directing Human Pluripotent Stem Cells Toward the Skeletal Muscle Lineage.

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Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) hold great promise as a potential source of cells for use in therapeutic medicine due to their ability to differentiate into cells from each germ layer and continuously self-renew. Despite this potential,
there remains great variation in both methodology and efficiency of generating mesodermal lineages from either hESCs or hiPSCs. Of particular difficulty has been the development of reliable protocols to efficiently differentiate skeletal muscle lineages. Although CD73+ mesenchymal cells have been obtained through sequential culturing and cell sorting methods, skeletal muscle cell yield was low and the protocol requires media for neuronal growth. Therefore we are currently establishing an alternative approach for directing differentiation of hESCs and hiPSCs toward the muscle lineage. To this end, when embryoid bodies (EB) derived from hESCs and hiPSCs were differentiated after 24 days, maximal enrichment for early stage or progenitor muscle markers was achieved compared to those differentiated up to 30 days. Subsequently, dissociated cells from EBs at each time point were sorted for either NCAM or M-Cadherin and expanded in standard culture conditions for promoting muscle cell growth. Both cell types were confirmed to express Pax3 via RT-PCR. Establishment of standard protocols for the reliable generation of muscle progenitor cells from PSCs will be important in future experiments for assaying the differentiation potential of Duchenne Muscular Dystrophy (DMD) patient-specific hiPSCs as well as for identifying the muscle cell type(s) needed for efficacious cell therapy.

428/B375
Abnormal Development of Skeletal Muscle Stem Cells in a Mouse Model of Spinal Muscular Atrophy.
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Spinal muscular atrophy (SMA) is the leading genetic cause of mortality in infants and toddlers. The disease is characterized by the degeneration of spinal cord motor neurons and muscular atrophy. SMA is caused by homozygous mutations in or deletion of the Survival Motor Neuron 1 (SMN1) gene, which results in low levels of SMN protein in all cells. Several lines of evidence suggest that muscle cells are themselves affected by the low levels of SMN and that they may indirectly contribute to poor motor neuron survival. Here we examined skeletal muscle precursor cells (SMPs), a subset of muscle satellite cells that are defined by their unique anatomical location beneath the basal lamina and adjacent to the muscle fiber plasma membrane. These cells play an essential role in muscle growth and repair. SMPs were isolated from skeletal muscle of postnatal day 2 SMA (Smn-/-; SMN2+/+) or wildtype (Smn+/+; SMN2+/+ or Smn+/-; SMN2+/+) animals using cell surface marker expression (Sherwood et al., Cell, 119 (2004), 543-554). We found similar numbers of SMPs in SMA and wildtype mice, at least at this stage of development. Further, at day 4 in vitro, the survival and proliferation of SMPs from SMA and wildtype mice were comparable. However, on day 4, SMPs from SMA animals appeared to have an accelerated differentiation, as revealed by the presence of more elongated cells and the premature expression of the muscle differentiation markers MyoD and Myosin Heavy Chain. These data suggest that early withdrawal of SMPs from the cell cycle may ultimately lead to a diminished number of these cells and, consequently, the reduced capacity for muscle growth, and potentially for muscle repair, observed in SMA.

429/B376
Differentiation of Adult Stem Cells Induces DNA Damage.
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It is established that multiple signaling pathways cooperate to maintain stem cell identity and, therefore, dysregulation of the tight control of these pathways will likely lead to changes in cell homeostasis. These changes can result in aberrant behavior and potentially transformation. Our lab, as well as others, has reported the transformation of stem cells following their implantation for the purposes of tissue regeneration. Herein, we report a putative mechanism to explain this
phenomenon. Preliminary experiments using a DNA integration method that induces GFP expression have revealed that DNA integration events increase when stem cells are subjected to differentiation signals. When adult muscle-derived stem cells (MDSCs) were treated to undergo either myogenic or osteogenic differentiation, we found that 1% and 3.5% of MDSCs that underwent osteogenic and myogenic differentiation, respectively, had integrated the reporter plasmid (over controls) after 7 days of culture. The concept of differentiation-induced DNA damage is not new. It was reported over 20 years ago that differentiation induces DNA nicking and strand breaks in a variety of cell types. To further investigate this, we focused primarily on myogenic differentiation. When we used a more refined fluorescence-activated cell-sorting based technique to explore DNA nicking, we found that, within 24 hours of myogenic differentiation, 12% of the cells had DNA damage. This trend increased over time, with the amount of damage peaking at 56% at 96 hours. By day 7, however, the damage was completely repaired. Furthermore, though we observed significant amounts of DNA damage, we observed no decrease in cell number, and no increase in the amount of dead/apoptotic cells over our controls. This data, though preliminary, provides evidence of transient DNA damage due to the differentiation of adult stem cells, and can help explain the spontaneous transformation of stem cells when used for regenerative medicine.

430/B377
Generations of Induced Pluripotent Stem Cells from Human Aortic Smooth Muscle Cells.
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Generations of induced pluripotent stem(iPS) cells have been intensively studied by a variety of reprogramming methods, but the molecular and functional properties of cells differentiated from iPS cells have not been well characterized. Here, we generated iPS cells from human aortic smooth muscle cells (HASMCs) by using lentiviral transduction of defined transcription factors. Established iPS cells were characterized to possess equivalent properties of human embryonic stem cells in terms of the cell surface markers, that is global mRNA and miRNA expression patterns, epigenetic status of OCT4, REX1, and NANOG promoters, and in vitro/In Vivo pluripotency. Our study reports, for the first time, the generation of iPS cells from HASMCs.

431/B378
SPARC and BMP2 Induce Cardiomyogenesis in Clonal Cardiac Stem Cell Lines in an Nkx2.5 Dependent Manner.
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Somatic stem cells of the heart have been isolated in past years by several groups using different strategies. These cell populations had different morphology but in common that they differentiate only to cells residing in the heart and that they could not be maintained as clonal stable cell lines so far. We have isolated and cloned stable cardiac stem cell lines from murine hearts and demonstrated that they express at the same time stemness genes such as Oct4, Nanog, Sox2, and Tert1, and marker genes for primitive mesoderm and early cardioblasts, such as Brachyury, Mesp1, Desmin, Nkx2.5, and Isl1. They self-renew in a LIF dependent manner for at least 80 passages without loosing their potential to spontaneously differentiate to cardiomyocytes, smooth muscle cells and endothelial cells, both in mono-layers and in embryoid body-like aggregates. Cardiomyocytes develop to mature pacemaker cells and atrial and ventricular cardiomyocytes expressing Connexin 43. SPARC, a matricellular protein promoting early cardiomyogenesis induces Nkx2.5 expression in cardiovascular progenitor cells and together with BMP2 promotes cardiomyogenesis via activation of the Nkx2.5 gene. Our results indicate successful isolation and long-term culture of clonal cardiovascular progenitor cells within a micro-environment accurately recapitulating a minimal stem cell niche.

432/B379
Mitochondrial ROS Formation Inhibit Cardiomyocyte Differentiation.
Reactive oxygen species (ROS) from different sources have direct or indirect effect on cardiomyocyte development. To investigate the effect of ROS produced within the electron transport chain (ETC) on cardiomyogenesis, embryonic stem (ES) cells expressing GFP under control of the cardiac specific α-myosin heavy chain (α-MHC) promoter were differentiated. Inhibition of complex III of the ETC with antimycin A or myxothiazol selectively abolished cardiomyocyte differentiation without effects on mesodermal or endothelial cell formation. However, ROS scavengers were able to reduce mitochondrial ROS levels and restored cardiomyocyte differentiation. We therefore conclude that the selective inhibition of cardiomyocyte differentiation was not due to impaired aerobic ATP production but to increased mitochondrial ROS levels. Cardiomyocyte formation was also restored by addition of a calcium-ionophore, indicating involvement of Ca²⁺ signaling. Our data were further supported by the finding that mitochondrial ROS impair Ca²⁺-spiking in early embryonic cardiomyocytes. Immunohistochemical analyses in differentiating ES cells revealed that mitochondrial ROS did not inhibit the expression of early cardiac transcription factors Nkx2.5 and MEF2C. In summary, elevated mitochondrial, but not cytosolic ROS levels block cardiomyocyte differentiation by inhibiting nuclear localization of early cardiac transcription factors via a Ca²⁺-dependent pathway.

433/B380
Cardiac Stem Cells Labeled with GFP Protein to Demonstrate Engraftment in Mouse Model as an Alternative Potential Cell Based Therapy for Cardiac Transplant Patients.

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Cardiovascular disease remains the leading cause of death worldwide. Acute ischaemic injury and chronic cardiomyopathies lead to permanent loss of cardiac tissue and ultimately heart failure. Current therapies aim largely to attenuate the pathological remodeling that occurs after injury and to reduce risk factors for cardiovascular disease. The current studies in animal models indicate that transplantation of cardiac stem cells has the potential to improve the function of ventricular muscle after ischaemic injury. The cardiac stem cell pool utilized in this study is cKit, KDR, Sca-1 (mice) Isl1 positive. The current experimental evidence suggests cardiac stem cells are able to engraft and differentiate into generation of new cardiac tissue. The predominant mechanisms of action of transplanted cardiac stem cells involve favorable physiological effects on injured myocardium. The adult heart possesses various pools of putative resident cardiac stem cells that can be isolated for therapy or manipulated In Vivo to improve the healing of cardiac muscle after injury as evident from this study. The genetic and protein profiles of the engrafted tissue when compared to the normal tissue were comparable. This study reveals the properties and potential utilization of cardiac stem cell populations for cardiac repair and regeneration.

434/B381
Human Fibroblast/Keratinocyte De-Differentiation into Human Cardiomyocytes in Tissue Culture.

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Somatic Cell De-differentiation provides an emerging strategy to produce embryo-independent pluripotent stem cells from somatic tissue. Induced pluripotent stem cells (iPC) demonstrate aptitude for de novo cardiac differentiation, yet their potential for heart disease therapy has not been tested. In this study, human fibroblasts/keratinocytes were transduced utilizing non-viral mechanisms with human stemness factors OCT3/4, SOX2, and c-MYC converted into an embryonic stem cell-like phenotype and demonstrated by their ability to spontaneously assimilate
into preimplantation host morula via diploid aggregation, unique to pluripotent cells. The iPCs were differentiated into normal heart tissue. The genetic and protein profiles of the differentiated cardiac patterning were comparable to normal human cardiac tissue. Human Fibroblasts / Keratinocytes De-differentiation with human stemness factors may provide an alternative potential to repair acute myocardial infarction, or in the understanding of novel drug discovery potential for heart disease.

**Focal Adhesion (435 – 447)**

**435/B382**

Proteomic Analysis of Myosin II-Mediated Focal Adhesion Maturation Reveals a Role for β-PIX in Relaxation-Mediated Rac1 Activation.

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Focal adhesions (FA) are plasma-membrane associated macromolecular assemblies that serve to physically connect cells to, as well as transduce signals to and from, the surrounding extracellular matrix (ECM). FA play a crucial role in the control of tissue structure and morphogenesis as well as cell motility. It is well established that FA undergo a tension-induced "maturation" process in which their size increases. Indeed, high cellular tension induced by Myosin II activity promotes FA growth and maturation, while reduced cellular tension promotes formation of small, immature FA. The protein compositional changes that accompany maturation of FA are thought to be critical to modulating signals transduced from the ECM that regulate cell growth and differentiation. To determine how FA protein composition changes during FA maturation, we developed a systematic method to isolate FA from human fibroblasts in native morphology, identify their protein composition by Mud-pit LC-MS proteomics, and validate the presence of specific proteins in FA by a series of stringent criteria. We performed this method to determine how FA-associated proteins respond to Myosin II activity to form FA structure by comparing the proteomimic profile of FA in the presence and absence of the Myosin II inhibitor blebbistatin. The results indicate that Myosin II activity promotes increased recruitment of a diversity of proteins in FA. Although Myosin II inhibition induces loss of many proteins from FA, a small subset of proteins actually increased in immature FA of blebbistatin-treated cells. Indeed, we found increased recruitment of β-PIX, a Rac1 GEF, to FA in blebbistatin-treated cells. We thus determine the role of β-Pix in Rac1 activation and lamellipodial protrusion that are induced by Myosin II inhibition. We found that overexpression of β-PIX increased the population of immature FA. In addition, when endogenous β-PIX was knocked-down, Rac1 activity induced by blebbistatin treatment was blocked, and the duration of FA maturation became shorter. These findings suggest that the β-PIX in FA is inhibited by Myosin II activity, and serves as a negative regulator of FA maturation to modulate Myosin II-driven FA maturation process.

**436/B383**

Cellular Junctions Are Dynamic and Undergo a Constant Change during the Development of the Drosophila.

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During development, cells migrate and change shape to form functional tissues and organs. To investigate cellular junctions in tissue movements and mechanics, we used GFP-fusions to follow zyxin, α-actinin, F-actin and zip/MyoII in Drosophila embryos. Zyxin-GFP outlined the cell membranes within amnioserosa and lateral epidermis and localized as puncta between junctions of 3 or more cells in the lateral epidermis. At the onset of dorsal closure, zyxin starts to accumulate in foci (larger, more intense puncta) at cell-cell junctions at the leading edge of the lateral epidermis, forming a beads-on-a-string pattern. Accumulation of zyxin coincides with the establishment of tension in the actin rich cable or purse string. Foci of zyxin-GFP or GFP-α-actinin can disappear, fuse or divide, suggesting that focal adhesions are dynamic and regulated.
during closure. After zippering forms a seam at each canthus, the purse string, and foci of zyxin and α-actinin disappear. This suggests that focal adhesions participate in dorsal closure but not in the mature epithelium, where we infer that cell-cell junctions predominate. Zyxin and α-actinin also localize where filopodia, extending from opposite sides of the leading edge during the zippering process, interact. Time-lapsed videos suggest that this occurs only in between the cells that are fated to zip together. In the myospheroid mutants, that lack zygotic βPS-integrin expression, zyxin still localized in foci along the purse string albeit with slight differences. Injection of latranculin-A disrupted zyxin foci. In embryos mutant for zip/MyoII, beads-on-a-string localization of zyxin is perturbed, suggesting a dependence on the tension along the purse string. When leading edge cells are ablated from the tissue using laser microsurgery, zyxin-GFP localizes to foci along the secondary purse string formed during the recovery. These data show that cell junctions undergo dynamic changes during development, indicate that junctions are closely associated with purse string, and that they respond to the tension developed in cells/tissues. Grant support: GM33830 to DPK and GM50877 to MCB

437/B384
Role of Tyrosine Phosphorylated Caveolin-1 in Caveolae Formation and Focal Adhesion Dynamics.
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Caveolin-1 (Cav1) is a major substrate of Src kinase, resulting in phosphorylation of Cav1 on tyrosine 14 (pY14Cav1). A phosphospecific antibody that localized pY14Cav1 to focal adhesions was subsequently found to crossreact with phospho-paxillin, questioning the focal adhesion distribution of pY14Cav1 (Hill et al, Traffic 8: 1695-1705, 2007). Metastatic MDA-435 tumor cells express limited endogenous Cav1 and few caveolae. To study pY14Cav1 distribution, we generated stable MDA-435 cell lines expressing RFP-tagged wild-type, phosphomimetic Y14DCav1, positively-charged Y14RCav1 and dominant-negative Y14FCav1 mutants. MDA-435 tumor cells stably transfected with either Y14D or Y14RCav1-mRFP showed a significant increase in caveolae formation with the latter having a pronounced effect on caveolae number, size and cluster formation. By TIRF microscopy wild-type Cav1-mRFP and Y14DCav1 did not colocalize with focal adhesion markers but were localized immediately behind the focal adhesion relative to the cell periphery. Y14FCav1-mRFP was not found in proximity to focal adhesions and Y14RCav1-mRFP clusters were further removed from focal adhesions compared to wild-type Cav1, implicating Y14 phosphorylation in the proximity of Cav1 clusters to focal adhesions. In both wild-type and Y14DCav1-mRFP stable MDA-435 cell lines, focal adhesions showed enhanced turnover relative to nontransfected or Y14FCav1 and Y14RCav1 transfected cell lines. Both Y14DCav1 and Y14RCav1 stable lines showed increased cell spreading with the Y14RCav1 stable line selectively presenting increased actin stress fibers. The similar effect of Y14 replacement by negatively charged aspartate (D) and positively charged arginine (R) amino acids on caveolae formation but not focal adhesion turnover suggests that tyrosine phosphorylation impacts on Cav1 function via both conformational and adaptor based mechanisms. The ability of wild type and phosphomimetic Cav1 to impact focal adhesion turnover in the absence of Cav1 recruitment to focal adhesions argues that tyrosine phosphorylated Cav1 indirectly regulates focal adhesion signaling and dynamics. Supported by CIHR grant MOP-43938.

438/B385
Cdk5 (Y15) Phosphorylation Is Required for Src Activation and Localization at Focal Adhesions.
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Purpose: We have recently shown that an intact phosphorylation site at Cdk5(Y15) is required for stress fiber assembly in spreading cells. The present study examines the possible role of Cdk5(Y15) in FA assembly. Methods: Human corneal limbal epithelial cells (HCLE) were plated on fibronectin and examined after 60min. Endogenous Cdk5 was suppressed by stable or
transient transfection with siRNA. GFP-tagged Cdk5, Cdk5(K33T), Cdk5(Y15F) were expressed by transfection; 15 microM olomoucine was used for pharmacological inhibition of Cdk5. Expression, localization, and protein-protein interactions of focal adhesion proteins were detected by immunofluorescence, immunoprecipitation, and immunoblotting. Subcellular fractions were isolated by differential detergent solubility. Results: Cdk5, Cdk5 (pY15), and p35 co-localized with vinculin and other focal adhesion (FA) proteins at FAs. Suppressing Cdk5 expression with siRNA prevented the increase in Src(pY416) normally associated with focal adhesion formation, blocked binding of Src to FAK, prevented Src-dependent phosphorylation of FAK(Y576) and paxillin(Y118), and reduced formation of vinculin-containing FAs (75.1%, p<0.05). Src levels in a detergent-insoluble cytoskeletal fraction were also decreased (39 %, p< 0.05), indicating that focal adhesions failed to link to the cytoskeleton. Although siCdk5 reduced focal adhesion formation, it did not block FAK(Y397) autophosphorylation or affect adhesion to fibronectin at 4oC, demonstrating that integrin engagement was not affected. Cdk5(Y15F) and Cdk5(T33K), Cdk5 mutants that can not be phosphorylated on Y15, mimicked the effects of Cdk5 siRNA on Src activation and focal adhesion formation. In contrast, olomoucine, which blocks Cdk5 activity without affecting Y15 phosphorylation, did not block Src activation or reduce focal adhesion formation, indicating that Cdk5 activity is not required. Conclusions: Expression of Cdk5 with an intact phosphorylation site at Y15 is required for Src activation and recruitment to focal adhesions in spreading cells. This function of Cdk5 is independent of its kinase activity.

439/B386

Regulation of Focal Adhesion Dynamics by Calpain Cleavage of FAK.

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Cell migration requires the coordinated and dynamic regulation of integrin-mediated adhesions. Previous studies in our laboratory have demonstrated that the limited proteolysis of talin1 by the calcium-dependent protease calpain 2 plays a critical role in focal adhesion disassembly in fibroblasts. We, and others, have identified another calpain substrate—the focal adhesion kinase (FAK)—which has also been shown to be a key component in the modulation of focal adhesion turnover. Using time-lapse video microscopy, we have examined the adhesion dynamics of GFP-talin1 in FAK-deficient cells. Quantification of adhesion assembly and disassembly rates demonstrates that FAK is required for the regulation of talin dynamics. However, the mechanisms by which FAK affects the adhesion dynamics of talin are not well understood. To begin to address this, we have mapped the calpain cleavage site of FAK and have generated a mutant form of FAK that is resistant to calpain proteolysis. Expression of wild-type but not calpain-resistant FAK restores focal adhesion dynamics in FAK-deficient cells. Current work is focused on elucidating how calpain cleavage of FAK contributes to the regulation of adhesion dynamics and cell migration.

440/B387

The Analysis of Force Dependent Zyxin Dynamics Using a Miniature Force Sensor.

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Cell migration requires generation and transmission of actin-myosin contractile force to the extracellular matrix via adhesive contacts. The assembly, disassembly, and size of focal adhesion plaques have been shown to alter traction force exerted by migrating cells. However, the mechanisms by which adhesive complexes regulate the transmission of traction force remain unclear. at focal adhesion sites, actin bundles also rapidly polymerize, which suggests that highly dynamic protein interactions and rapid actin assembly may be tightly coupled to the regulation of traction force at adhesive contacts. The focal adhesion protein zyxin recruits Ena/VASP proteins to promote actin polymerization, therefore, zyxin may be a key regulator of traction force. Using an elastic micro-pillar substrate and epithelial cells stably expressing GFP-tagged zyxin, we found the intensity of zyxin-GFP closely followed increasing and decreasing forces measured by
individual force-sensing pillars. Treatment with Rho kinase inhibitor Y27632 resulted in dissipation of zyxin-GFP from adhesion sites and reduction in traction force, suggesting that activated Myosin II is required for the zyxin localization. Surprisingly, the suppression of zyxin by siRNA resulted in faster single cell migration, but did not affect cell sheet migration in wound healing assays. This may be due to the presence of cell-cell adhesion, but further studies are needed to tease out the effect of zyxin siRNA on cell-cell adhesion. Because of its ability to accumulate at force-bearing adhesion sites and effect on single cell migration, zyxin plays a critical role in the regulation of traction force.

441/B388

Vinculin Stabilizes Nascent Adhesions and Establishes the Lamellipodium-Lamella Border in Migrating Cells.

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The actin cytoskeleton at the leading edge of migrating cells consists of two actin networks, the lamellipodium (LP), characterized by fast polymerization-driven retrograde actin flow and the lamella (LM) with slow Myosin II (myoII) mediated actin flow. The engagement of LP actin to the ECM via nascent integrin-mediated focal adhesions (FA) establishes the flow velocity gradient between LP and LM. Nascent adhesions then elongate and mature via myoII LM actin flow. How integrins are connected to the retrograde actin flow is not known. Using primary murine embryonic fibroblasts (MEF) with cre-mediated excision of the vinculin gene (Vcl), we sought to test the hypothesis that vinculin mediates the coupling of actin retrograde flow to the ECM in FA. Single Vcl-/- MEF migrated faster than control (Vclfl/fl) MEF and displayed impaired anisotropic spreading. To determine if LP/LM organization was affected by loss of vinculin, we analyzed distributions of phospho-myosin light chain (pMLC), cortactin, and paxillin. This revealed a shift in pMLC distribution towards the cell edge, reduced LP paxillin intensity, and broadening of the cortactin band at the leading edge, suggesting a loss of delineation between the LP and LM. To test this, we performed spinning disc confocal (SDC) microscopy of MEF containing fluorescent paxillin and actin. This revealed a reduction in the rate of formation of short-lived, diffraction limited FA in the LP of Vcl-/- MEF, indicating an impaired stabilization of nascent LP FA. Kymograph analyses of high resolution DIC and quantitative fluorescent speckle SDC microscopy of actin indicate the lack of two distinct velocity zones of retrograde f-actin flow near the leading edge and an increased retrograde flow velocity in the LM region of Vcl-/- MEF. We suggest that vinculin stabilizes nascent FA by coupling to lamellipodial actin flow, thus establishing the flow velocity gradient between LP and LM and promoting the maturation of nascent adhesions. This implicates vinculin as an essential component linking the dynamic actin cytoskeleton to the ECM during cell migration.

442/B389

Analysis of Traction Stress Variation across Single Focal Adhesions.

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The ability of eukaryotic cells to generate force and to sense the mechanical properties of the extracellular matrix (ECM) underlies many biological processes, such as cell migration, proliferation and differentiation. This is achieved in part by integrin-mediated focal adhesions (FA), protein assemblies that couple contractile actomyosin bundles to the plasma membrane and transmit force generated by the cytoskeleton to the ECM. It has been demonstrated recently that protein composition and/or post-translational modification state can vary across an individual
FA. However, whether this translates to variation in physiological properties and/or function for sub-FA domains is not known. We used dynamic high-resolution traction force microscopy (Sabass et al., 2008) in migrating fibroblasts to analyze the distribution of traction stresses on the ECM along single FA and to correlate it with the organization of specific proteins within FA. We identified two distinctive patterns of traction stress in single FA at protruding cells edges: for the majority of FAs, the region of maximum stress was located at the geometrical center of the FA and colocalized with maximal paxillin-eGFP intensity. For a subset of FA the traction peak was skewed significantly toward their distal edges (cell periphery). Translocation of the traction force maximum from the FA center towards its distal edge occurred abruptly and did not depend on the functional state (growing, stationary, sliding or disassembling) of FA. We then tested the hypothesis that variation in force transmission across a FA is directly coupled to differences in biochemical composition across an individual FA. We found that expression of paxillin mutants that perturb the gradient of paxillin phosphorylation across FA significantly reduced the traction and constrained the traction distribution to the center of FA. However, the latter effect could be rescued by decreasing myosin contractility. These results suggest that the magnitude of traction stress is modulated by local changes in phosphorylation state of paxillin while traction oscillation along the FA is purely a stochastic process mediated by balance between cellular contraction and ECM stiffness.

443/B390
Mechanical Regulation of Focal Adhesions with Spatial Constraints.
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The regulation of cellular traction stresses impinged on the extracellular matrix is essential in cell adhesion and migration as well as remodeling of the extracellular matrix. Adherent cells engage with the surrounding extracellular matrix (ECM) through dynamic protein assemblies termed focal adhesions. The size of the focal adhesion plaque can be intimately linked to changes in intracellular or extracellular tension. Newly formed adhesion clusters near the leading edge of cells are quite small (<1 \( \mu \)m\(^2\)) and generate very little stress on the ECM. If tension is applied, these plaques increase in area and, subsequently, the force impinged on the extracellular matrix increases. Because of the co-dependency of these two parameters, it is unknown whether increased focal adhesion size is required for the generation of large cellular traction stresses. We utilized microcontact printing to spatially control the distribution of ECM ligands to constrain focal adhesion area while simultaneously measuring cellular traction stresses impinged on the ECM using high resolution traction force microscopy. U2OS osteosarcoma cells and NIH 3T3 fibroblasts successfully spread on an array of 1 \( \mu \)m fibronectin circles (1 \( \mu \)m in diameter) with an approximate edge to edge spacing of 2 \( \mu \)m. Focal adhesions localize to the micropatterned spots of fibronectin and are constrained to 1 \( \mu \)m\(^2\) in area, approximately 35% of the size typically observed on unpatterned substrates. The focal adhesion proteins vinculin and zyxin, which are known to localize to focal adhesions at the end of their growth cycle, localize to constrained focal adhesions. The median traction stress exerted at these focal adhesions is typically 66 Pa, similar to control cells. However, the large variance of traction stress observed demonstrate that focal adhesions of similar size do not exert identical stress. Application of external force to constrained focal adhesions indicate a diminished rupture stress, as compared to unconstrained adhesions. Thus, the mechanical behavior of constrained focal adhesions retains some characteristic of unconstrained adhesions, but appear less responsive to changes in their external environment.

444/B391
Cell Geometry Regulates Traction Force Generation.
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Over the past decade, it has become increasingly clear that mechanical forces regulate many critical aspects of cellular functions from proliferation, differentiation, motility, to tissue formation.
Cells can respond to not only active forces exerted on them but also the resistance to forces they exert on the environment, termed traction forces. Although traction forces are known to be affected by cell geometry, the underlying principles governing geometric control of traction forces remain unknown. The purpose of this study was to determine these principles. A novel technique was first developed to generate high quality adhesion patterns on polyacrylamide hydrogels. This technique involves printing activated adhesion proteins onto a glass coverslip in a defined pattern, which is then transferred to the gel during polymerization. Combining patterned hydrogels with traction force microscopy then allows the measurement of traction forces as a function of cell area and shape. Our results suggest that traction forces of NIH 3T3 cells are regulated primarily by the distance from the center of the cell to the periphery. Forces are concentrated at distal areas and correlated positively with the distance from the cell center. This relationship remains intact until the cell takes an exceedingly elongated shape, where traction forces appear to become limited by reduction in focal adhesion size. These results demonstrate the dependence of cell traction force generation on cell shape, in a manner similar to the dependence of tension on the shape of a spread liquid drop. Resistance to these forces may in turn stimulate mechanotransduction responses that regulate a wide range of cell functions.

445/B392
The Role of Mechanical Force in Regulation of Focal Adhesion Complex Proteins in Developing Kidney.
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Mechanical force has been shown to affect the expression of focal adhesion complex proteins such as focal adhesion kinase (FAK). Our previous studies have found that focal adhesion kinase (FAK) was degraded gradually in late embryonic rat kidney development and rapidly diminished after birth, yet the regulating mechanism was unclear. We hypothesized that degradation of FAK in the developing kidney is caused by urine shear flow mediated by primary cilium of epithelium. This triggers elevation of intracellular calcium concentration and activates calpain that further cleaves FAK. Immunohistochemistry staining showed that cilia formation was found in kidney tubules at embryonic day 19.5 and later during development, which matched our initial observation in FAK degradation in embryonic kidney. In order to mimic urine flow in the kidney, we applied luminal shear flow (4 dyne/cm²) to MDCK subline 3B5 cells. We observed degradation of focal adhesion complex proteins and degraded fragments of α-spectrin at 145 and 120 kD within 30 min. These results were similar as in the developing kidney, suggesting possible activation of calpain and caspase-3. on the other hand, bidirectional and rotational stress produced by placing cultured cells on a rotator or shaker at 20 rpm resulted in downregulation of FAK in time dependent manner. Immunofluorescence studies showed that as fluid shear stress increased (2, 4, 6 dyne/cm²), FAK translocated from cell periphery to cytosol. The final localization of FAK was similar to that of developing kidney. This study provided a possible mechanism on how mechanical force regulates focal adhesion complex proteins during kidney development.

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The Regulatory Role of Clusterin Expression in the Intracellular Metastatic Signaling of Prostate Cancer Cells.
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Acquisition of migratory capacity of prostate cancer cells is an essential event for metastatic disease progression; however, the molecular mechanism underlying acquisition of a metastatic capacity remains unresolved. Clusterin (CLU) is a secreted chaperone protein, over-expressed in many cancers that has been previously reported as up-regulated during Castration Resistant
progression of prostate cancer (CRPC). We used an antibody array to identify changes in protein expression and phosphorylation of PC3 prostate cancer cells in which CLU expression was suppressed by siRNA knockdown. We observed that CLU siRNA knockdown leads to decreased focal adhesion kinase (FAK) phosphorylation as well as its downstream targets. FAK is a member of a family of non-receptor protein-tyrosine kinases that acts as a key regulator of cell migration and whose expression level correlates with CRPC progression. Validating the antibody array results, we confirmed that CLU siRNA knockdown decreases FAK phosphorylation in PC3 cells without affecting total FAK levels by immunoblot analysis. We have gone on to show that CLU siRNA treatment suppresses serum- and VEGF-inducing FAK phosphorylation, and attenuates PC-3 cell migration and invasion capacity in wound healing and matrigel invasion assays. All together, these observations implicate CLU as an important regulator of cell motility and FAK activation in PC3 cells.

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Structure, Function, and Evolution of Cell Adhesion Proteins.
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Phylogenomic analyses have revealed that choanoflagellates are a sister group to the Metazoa. Although choanoflagellates are predominantly unicellular, the genome of Monosiga brevicollis contains genes for proteins that are essential for cell adhesion. These include cadherins and several other cytoskeletal proteins associated with adherens junctions. M. brevicollis also contains a single gene for the large, modular protein talin, which is essential for the assembly of focal adhesion complexes in animal cells. Talin is also required for cell adhesion and cell differentiation in the amoebozoan Dictyostelium discoideum. The presence of talin in amoebozoans, choanoflagellates, and metazoans (including Trichoplax adhaerens) indicates that talin was present in the last common ancestor of the unikonts (amoebozoans, animals, choanoflagellates, and fungi). Talin is not present in fungi, but the essential, conserved C-terminal THATCH (I/LWEQ) domain that mediates F-actin binding and subcellular targeting in talin is present in the fungal cytoskeletal protein Sla2p/End4p. As part of an effort to understand the structure, function, and evolution of multicomponent assemblies required for cell adhesion, we have begun the study of talin and its physiological partner vinculin from M. brevicollis. The structure and regulation of the conserved C-terminus of M. brevicollis talin is similar to that of vertebrate talins. M. brevicollis vinculin shares several conserved features with animal vinculin, but the intrasteric regulation of vinculin is likely to be different in M. brevicollis. We anticipate that comparison of choanoflagellate adhesion proteins with those from metazoans will improve our understanding the evolution of these modular proteins as components of multiprotein assemblies and identify critical factors in the origins of multicellularity in animals.

Organization and Regulation of the Extracellular Matrix (448 – 465)

448/B395

Cellular Contractility Modulation of Extracellular Matrix Assembly, Composition and Growth Factor Activation in Human Mesenchymal Stem Cells.
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Mesenchymal stem cells (MSC) may hold great therapeutic promise, but like most other cells, MSC possess a contractile cytoskeleton which they constantly employ in shaping and responding to their microenvironment. MSC secrete a wide variety of extracellular matrix (ECM) proteins, from major structural proteins such as collagens and fibronectin to small, growth factor-binding proteoglycans. As ECM composition continues to emerge as a major cue in the regulation of biological processes, the extent to which MSC secretory patterns might coordinate cell growth and differentiation is important to understand. Furthermore, the TGF-beta family of growth factors are known to bind ECM and regulate differentiation. By manipulating the stiffness of cell culture substrates and thus the contractile engagement of MSC cytoskeleton, we find within hours that
marked differences in morphology as well as focal adhesion formation are evident. MSC assembly of Fibronectin (FN) is increased from ~5h up to 2 weeks when cells are plated on stiff substrates. One implication of such early-onset matrix assembly differences is the modulation of TGF-beta growth factor activation by MSC, and our data indeed indicates that the level of active TGF-beta protein is higher in MSC cultured on stiffer substrates. Ongoing work aims to specifically determine how FN dynamics correlates with TGF-beta activity and also to correlate broader ECM roles in MSC lineage choices.

449/B396
Signal Transduction Mediates the Remodeling of the Stem Cell Microenvironment.
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The field of stem cell biology has immense potential but there is a lack of understanding about the relationship between changes in stem cell behavior and remodeling of the stem cell niche which prevents the realization of that potential. Identifying signal transduction pathways that activate the remodeling of collagen will help provide the information necessary to close some of the information gaps in stem cell biology. To address this problem, we monitored stem cell gene expression levels along with differential collagen gel compaction rates in changing three dimensional environments and we used three dimensional image analysis to provide additional spatial information. There is a correlation between signal pathway activities and collagen remodeling reflected in the level of gel compaction and collagen fiber organization. This relationship is also reflected by internal reorganization of the actin cytoskeleton. These results suggest that specific protein activation states and actin filament arrangement impacts the organization of collagen in three dimensions.

450/B397
Behavior of Cells on Tissue-Like Collagen Matrices.
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Using a novel computer controlled process, we have prepared sheets of collagen from soluble collagen which have skin-like, tendon-like, or an aligned braided structure on glass and plastic substrates. The process provides uniform structures over large area as confirmed by AFM, SEM, laser diffraction, and polarized microscopy. In some experiments, parallel microgrooves have been created during the deposition process while preserving fibril structure. Various cell types when plated on these matrices preferentially align and migrate along the fibrils or along the crimped ridges depending on the geometrical parameters of the surface nanostructure, including fibroblasts, stem cells (induced pluripotent, adipose-derived and bone marrow-derived), cortical nerves, periodontal ligament fibroblasts and osteosarcoma cells. Over the course of a few days myoblasts fuse to form aligned myofibrils. Cells cultured on collagen scaffolds were monitored by light microscopy and analyzed for patterns of protein and gene expression by immunofluorescent staining and qPCR. Primary corneal keratocytes align along fibrils and show gene expression more characteristic of a differentiated phenotype vs. phenotype observed in wounded cornea, suggesting that this collagen scaffold may improve the quality of the corneal wound repair. In addition, supplementing collagen scaffolds with a variety of growth factors (attached via glycosaminoglycans) induces faster cell migration and show a potential for a more controllable cell growth system in vitro.

451/B398
Coupled and Integrated Spatiotemporal Responses of Fibronectin to Mechanical Stimulation.
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The extracellular matrix (ECM) is a critical cellular component for a variety of reasons including its structural support. Fibronectin (FN) is an ECM protein that is secreted by cells as a soluble dimer and assembled into insoluble multimeric fibrils at the cell surface. This extracellular component has been linked to wound healing, cell adhesion, blood coagulation, cell differentiation and migration, maintenance of the cellular cytoskeleton, and tumor metastasis. In addition FN is also highly elastic and constantly subjected to cell-generated forces. We believe that there is an intricate coupled feedback system between the ECM and mechanical stimulation. Also, since cells alter their extracellular environment through FN modulation and secretion, we investigated spatiotemporal alterations of the extracellular environment by cells in response to applied mechanical stimulation. In this study we used a custom fabricated device to expose single cells to equibiaxial strain and fluid shear stress for a period of up to 24 hours. We used immunofluorescence to examine the effects mechanical stimulation had on intracellular and extracellular FN patterns. After mechanical stimulation immunofluorescence images revealed an increase of extracellular FN matrix fibrils relative to unstimulated cells. Intracellular FN patterns were also observed to localize around the cell periphery in response to mechanical stress. The coupled stimulation and feedback from cells in terms of ECM and mechanical stimulation were probed to understand the intricate balance in mechanobiology. We believe that these results will be of great significance to the field of matrix biology and have implications into various ECM related fields such as wound healing and cell adhesion.

Fibronectin (FN) is an essential component of the extracellular matrix (ECM) and is assembled into a fibrillar matrix by many cell types. This large multi-domain protein harbors binding sites for cell adhesion receptors, ECM components, and FN itself. FN fibril assembly is initiated by integrin receptor binding to the III 9-10 domain, while fibril deposition depends on FN binding to the III 1-2 domain. We have examined the feasibility of directing FN assembly by using surface chemistry to conjugate these domains, individually or together, to synthetic polymers. Bacterial expression was used to prepare GST fusion proteins containing III 1-2, III 9-10, or III 1-2-III 9-10 expressed as a single polypeptide. We also prepared GST-III 1-2 Mut-III 9-10, which contains mutations that disrupt a salt bridge between III 1 and III 2 and enhances FN binding activity (Karuri et al. (2009) J. Biol. Chem. 284, 3445-52). A modular approach was used to covalently link domains to a surface metallic complex of zirconium and carbamate groups on polyurethane through phosphonic acid and chloroformate cross-linkers. Surface chemistry was evaluated at each step by contact angle measurements, and ELISA results confirmed that equivalent amounts of FN domains were coupled to the surfaces. Little cell attachment was observed on III 1-2 alone. Interestingly, while surfaces with III 9-10 supported cell attachment, the inclusion of the III 1-2 domain, either as a mixture or a fusion with III 9-10, significantly increased FN fibril assembly. Moreover, surfaces conjugated with III 1-2 Mut-III 9-10 supported more fibril assembly than III 1-2-III 9-10. These data suggest cooperation between surface-attached III 9-10 and III 1-2. III 9-10 supports cell adhesion, while III 1-2 appears to provide tethering sites for cell-associated FN which facilitates conformational changes during fibril assembly. Thus attachment of these domains creates a bioactive surface for matrix assembly and suggests a new avenue for directing FN matrix assembly on biomaterials.

Fibronectin elasticity does not involve unfolding of type III domains.
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Fibronectin (FN) fibrils are elastic and can be stretched by cells to four times their resting length. The origin of this elasticity has been debated: some posit that the elasticity is mediated by unfolding of the 15 tandem Type III repeats, each of which has a similar, 7-beta-strand structure. Others believe that the elasticity is a function of a transition from a compact quaternary conformation to an extended conformation of the entire dimer. Here we investigate the folded state of the Type III domains within fibronectin fibrils by labeling free cysteines in assembled fibrils with a thiol reactive fluorescent probe. In this protocol, buried cysteines are not labeled by the probe, while exposed cysteines are. NIH3T3 cells were plated onto glass coverslips and allowed to form fibrils for 24 hours, after which time the fibrils were labeled with the thiol reactive probe. Initial results showed that neither of the native free cysteines (buried within domain III-7 and domain III-15) were exposed in assembling fibrils. Subsequently, 15 recombinant FNs were engineered such that each contained only a single, buried free cysteine within a Type III domain. Results show that only a small subset of these recombinant FNs could be labeled with the thiol-reactive probe. This indicates that most Type III domains remain folded during fibronectin fibril assembly. Furthermore, the few domains that were labeled during fibril growth are consistent with previous work that has shown that these domains spontaneously unfold in solution, independent of applied force. Since the observed 4-fold extension of fibronectin fibrils would require complete unfolding of all 15 domains, this data suggests that this cannot be the primary mechanism of fibronectin elasticity.

454/B401
A Proteomics Study of Molecular Interactions That Lead to the Establishment and Stabilization of a Mature Fibronectin Matrix.
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Fibronectin fibrillogenesis is a complex, multi-step process that involves the conversion of soluble, compact fibronectin into an insoluble fibrillar matrix. The formation, extension, and augmentation of fibronectin fibrils represent essential steps in the establishment of a mature matrix that is biochemically characterized by its insolubility in the detergent deoxycholate (DOC). These fibrils are formed by various intermolecular fibronectin-fibronectin interactions involving specific domains within fibronectin. Although several fibronectin self-binding sites have been identified, the domains responsible for matrix maturation are largely unknown. In this study we used mass spectrometry (MS) in a “bottom-up” strategy to identify regions of fibronectin that are involved in the establishment and stabilization of the mature matrix. Extended proteolysis of fibronectin matrices from NIH3T3 fibroblasts yielded multiple protease-resistant fibril fragments that were insoluble in DOC. Proteomic analyses identified three non-overlapping domains, each composed of type III modules, that were common to most of the DOC-insoluble fibril fragments analyzed by MS. Interestingly, none of the fragments contained the III1-2 domain of fibronectin which is known to be required for matrix maturation. The molecular weights of the proteolytic fragments ranged between 10 kDa and 50 kDa and were substantially less than the predicted size of a contiguous fragment bearing all three domains (142.5 kDa). Preliminary binding studies indicate that two of these domains contain fibronectin self-interacting sites. Based on these results, we propose that these domains are involved in fibronectin-fibronectin intermolecular interactions required for DOC-insolubility and matrix fibril stability.

455/B402
A Cellular Reporter for Type I Collagen Transcription Allows Optimization of Bioartificial Tissue Engineered Arteries.
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Objective: Optimizing collagen content of tissue-engineered arteries

Methods: Primary neonatal rat vascular smooth muscle cells (vSMC) were stably transfected with a plasmid containing the human COL1A1 promoter upstream of the luc2 gene. Three-dimensional fibrin-based tissue constructs were then fabricated in adherent disc and mandrel-mounted tubular geometries. Constructs were incubated for varying times in experimental media or in a bioreactor to apply cyclic distension and were assayed noninvasively for luminescence periodically. This luciferase strategy predicts changes in both collagen transcription and deposition (Weinbaum JS, Qi J, Tranquillo RT, Tissue Eng. Part C, 2009).

Results: Decreasing the concentration of the fibrinolytic inhibitor ε-ACA to 4 mM (from the 8 mM standard concentration) increased the collagen transcription rate in the disc constructs by 25±4% while raising the concentration to 16 mM lowered transcription by 37±4%. Inhibition of ERK signaling by the addition of 5 and 10 μM PD98059 lowered the transcription rate by 44±6% and 67±2%, respectively. Reduction of TGF-β from 1 to 0 ng/ml also led to a decrease in the transcription rate by 63±9%. In tubular constructs, TGF-β also showed potency, increasing the collagen transcription rate an average of 3.7±1.2 fold. Cyclic distension of the tubular constructs along with TGF-β supplementation increased the collagen transcription rate by 4.4±1.6 fold. Conclusions: vSMC engineered to report type I collagen promoter activity compact and remodel fibrin gel into tissue constructs and respond to extracellular and intracellular stimuli with easily monitored collagen transcription rates. These results suggest that a combination of minimal fibrinolytic inhibition, TGF-β supplementation, amplification of ERK signal, and mechanical conditioning may lead to improved vSMC-based tissue-engineered arteries; ideally, this approach will enable culture optimization leading to a bioartificial artery with sufficient mechanical strength to withstand pulsatile blood flow.

456/B403
TGF-β3 Regulates Fibrosis in a 3D Self-Assembled Corneal Matrix Model.
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Objective: Corneal injury/wounding, leads to death in cells adjacent to the wound, and stimulates cells distal to the wound to differentiate into fibroblast and/or myofibroblast phenotypes that orchestrate proliferation, cell migration and matrix remodeling. Scar formation is a common output of these injuries; however, the regulatory factors controlling the matrix remodeling are not well understood. Methods: TGF-β is known to modulate collagen synthesis and cause tissue fibrosis. We have developed an In Vitro 3D self-assembled model using human corneal fibroblasts in order to investigate the effect of three different TGF-β isoforms over time. Constructs were stimulated with stable Vitamin C (VitC) derivative and TGF-β1, -β2, and -β3 (T1, T2 and T3) at 0.1ng/ml concentration for 4 and 8 weeks. Results were analyzed using immunofluorescence and electron microscopy. Results: All TGF-β isoforms stimulated an increase in construct thickness by at least 3 fold, compared to controls (no TGF-β). High amounts of collagen III were shown for both T1 and T2 where T3 showed no expression. When smooth muscle actin (SMA) was localized, numerous cells were SMA-positive in T1, T2 and the controls. However, T3 had far fewer SMA-positive cells. Cells in T1, T2 and T3 expressed EDA-Fn at 4 weeks. No EDA-Fn was observed at 8 weeks. The presence of low numbers of myofibroblasts in T3 was confirmed by TEM results, suggesting a non-fibrotic TGF-β isoform. Finally, collagen fibril diameters were similar to In Vivo with T3; whereas, they were smaller in control, T1 and T2. Conclusions: Overall, our data demonstrates that T3 stimulates the deposition of a self-assembled matrix to the same level as T1 or T2, however, the response is significantly different than T1 or T2 in that, no expression of specific fibrotic markers was observed.

457/B404
TGF-β Leads to Diminished Collagen Deposition via Decreased Perk during Long-Term Cyclic Stretching of Engineered Connective Tissue.
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Objective: Optimize growth conditions for engineered tissue. Methods: Tubular constructs (TC) were formed by entrapment of neonatal human dermal fibroblasts in fibrin gel cast around a mandrel. TC were incubated in HG-DMEM supplemented with 10% FBS, +/- 1 ng/ml TGF-β, 2 μg/ml insulin and 50 μg/ml ascorbic acid. After 2 wk static incubation, TC were subjected to cyclic distention (CD) for 3 or 5 wk then tested for ultimate tensile stress (UTS) and modulus (E), cell (via DNA), collagen (via hydroxyproline), and total protein contents. Results: At 2 wk, +TGF-β yielded a 101% higher collagen density but no difference in UTS/modulus compared to -TGF. at 5 and 7 wk, -TGF-β yielded higher UTS/modulus and collagen density (also better organized by picrosirius red staining), but lower elastin density compared to +TGF-β. Western blots at 5 and 7 wk showed increased p-ERK for -TGF-β, correlating with higher collagen density. Western blots for αSMA and SMAD2/3 revealed 16x and 10x higher levels for +TGF-β. CD experiments for 5 wk were conducted -TGF-β to improve tensile properties (UTS = 1.4±0.3 MPa, E = 2.5±0.6 MPa), then +TGF-β for 2 wk to increase elastin density (0.19±0.1 mg/mL). Conclusions: While TGF-β can enhance elastin production, it is detrimental to collagen deposition during CD, attributable to inhibition of p-ERK. However, TGF-β following CD can yield both physiological UTS and modulus and substantial elastin content.

458/B405
SHG Imaging of Collagen Reorganization during Cervical Remodeling.
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The ability of the cervix to dilate during labor is the culmination of a series of extracellular matrix remodeling events that begin early in pregnancy. The processes underlying cervical remodeling are still not clearly understood. In this study we quantitatively assess the changes of collagen structure via second harmonic generation (SHG) imaging of murine cervix during cervical remodeling. Cervices were collected from pregnant mice on day 6, 12, 15, and 18 of the 19-day mouse gestation, frozen in OCT medium and sectioned at 50um. Regions of the stroma near the cervical os were excited at 900nm and SHG signal was detected at 450nm, using a Zeiss LSM 510 NLO confocal microscope. Images collected in both the forward and backward directions were analyzed and quantified for changes in collagen fiber diameter, porosity (space between fibers), overall intensity and forward to backward intensity ratio (F/B), using ImageJ. Visual inspection of the images revealed substantial changes in collagen morphology throughout gestation. Early in pregnancy, fiber bundles appeared relatively short, narrow and straight, progressing to large, highly "crimped" fiber bundles by day 18. The amount of open space between fibers appeared to increase and was maximal at the end of pregnancy. The overall mean intensity of SHG signal increased quantitatively throughout gestation in both directions up to day 15. on day 18 the intensity in the backward direction decreased, resulting in an increased F/B ratio. This suggests increased randomness in the collagen and/or changes in the scattering properties of the extracellular matrix as a whole by day 18, both of which can lead to decreases in backward SHG. Fiber diameter progressively increased 30-40% from early to late pregnancy. Pores between collagen fibers increased in diameter by 40-70% and in spacing by 30-50%. However, the number of pores decreased by 50% resulting in no net change in fractional area. The dramatic collagen reorganization revealed by these studies may account for changes in tissue mechanics and loss of tensile strength during cervical remodeling.

459/B406
The Association of Endurance Training with Heteropterys aphrodisiaca Results More Resistent Tendons to Support High Loads from Intense Exercises.
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Popular Brazilian medicine uses *Heteropterys aphrodisiaca* infusion as a tonic or stimulant, for the treatment of nervous debility and breakdown and for muscle and bone weakness. The present study investigated the effects of *H. aphrodisiaca* infusion on the tendon properties under endurance training. Forty-eight male rats were divided into four groups (n = 12): CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT-control trained, HT- *H. aphrodisiaca* trained. The training protocol consisted in running on a motorized treadmill, 5 times a week, with weekly increase in treadmill velocity and duration, consisting in 3 weeks of adaptation and 8 weeks of training. Control groups received water while the HS and HT groups received *H. aphrodisiaca* infusion (dose: 35 mg/animal/day) by gavage during 8 weeks of training or sedentary period. Achilles tendons were frozen for biochemical and biomechanical analysis or preserved in Karnovsky's fixative, then processed for histomorphological analysis by conventional and polarized light microscopy. Biomechanical analysis showed significant increase in maximum stress and modulus of elasticity of the tendons of the HT animals. The metalloproteinase-2 activity was reduced in the HT group. The compression region of tendons of HT animals had a stronger and more intense metachromasy, which suggests increase in glycosaminoglycan (GAG) concentration in this region of the tendon, although the GAG levels were statistically similar among the four groups. The most intense birefringence was observed in the tendon of HT animals, which may indicate a higher organizational level of collagen bundles. The quantification analysis of hydroxyproline in the different groups showed a higher content in the HT group. Our results suggest that the association of endurance training with *H. aphrodisiaca* resulted in more resistant tendons to support high loads from intense muscle contraction. This association stimulated tendon remodeling, promoting higher collagen content and probably increased cross-links. Despite the clear anabolic effects of *H. aphrodisiaca* and the endurance exercise association, no side effects were observed, such as those found for synthetic anabolic androgenic steroids.

**460/B407**

**Periostin Knockout Mice Show a Reduction in Collagen Production, Wound Contraction and TGF Beta 1 Response in Experimentally Induced Dermal Wounds.**

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Periostin is a recently identified member of the matricellular proteins family, proteins identified by their ability to mediate interactions between cells and the extracellular matrix (ECM). The recently developed periostin knockout mouse shows serious alterations in the structure of the extracellular matrix (ECM) in skin, where decreased collagen fibre diameter is evident. Evidence Periostin may be important for the dermal wound repair, but over-expression of periostin is associated with keloid and hypertrophic scarring, conditions caused by excessive extracellular matrix production and prolonged survival of smooth muscle actin-rich myofibroblasts. We have previously shown that periostin is expressed in granulation tissue formed after excisional wound repair, where it is associated with myofibroblasts. The aim of this investigation was to examine dermal wound repair in periostin knockout mice. An excisional cutaneous punch biopsy model was employed with C57BL/6 wild-type (WT) and sex matched littermate periostin knockout mice. Repair was assessed In Vivo at 3, 7 and 11 days post-wounding through immunohistochemistry (IHC), and calculation of wound area. In periostin knockout mice, a delay in wound area reduction starting at day 3 was observed, increasing through to day 7 in comparison with WT mice. Analysis of collagen using Masson's trichrome staining revealed a reduction of collagen in periostin knockout mice in the granulation tissue at day 7 in the periostin knockout mice, which was quantified using hydroxyproline assays (p<0.05). In Vitro culture of periostin knockout and wild-type dermal fibroblasts demonstrated that cells from periostin knockouts showed reduced mRNA levels of collagen, SMA and connective tissue growth factor in response to transforming growth factor (TGF) beta 1. Furthermore, the cells were unable to contract collagen gels in comparison with wild-type cells (p<0.01). We conclude that periostin mediates dermal fibroblast response to TGF beta 1 and plays a central role in dermal wound repair.
POMGnT1 Deficiency Affects Assembly of the Basement Membrane.
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The pial basement membrane (PBM) is a thin sheet of extracellular matrix (ECM) formed between the glia limitans and the meninges. Formation of PBM is regulated by cellular receptors of ECM such as α-dystroglycan and integrins. α-dystroglycan is a heavily glycosylated protein that binds to laminin of the basement membrane. It is modified by several glycosyltransferases, which when mutated causes congenital muscular dystrophies (CMDs) with type II lissencephaly. One of the glycosyltransferases is POMGnT1, protein O-mannose N-acetylgalcosaminyltransferase 1. Analysis of POMGnT1 knockout revealed that breaches in the PBM is the key initial event that results in neuronal overmigration, leading to type II lissencephaly. The mechanisms of PBM breaching are poorly understood. We hypothesized that assembly of pial BM is reduced in rate. To test this hypothesis, we analyzed assembly of extracellular matrix on neural stem cells (NSCs) isolated from POMGnT1 knockout mice. Laminin or Matrigel were added to cultured neural spheres. ECM aggregation on neural spheres was detected by immunofluorescence staining against major components of the basement membrane. When incubated with Matrigel, the ECM aggregates were composed of all four major components of the basement membrane. Overall ECM aggregation was reduced on POMGnT1 knockout neural spheres when compared to the wildtype. Further analysis showed that aggregation on POMGnT1 null neural spheres had reduced rate as revealed by slower growth in size of aggregates compared to the wildtype. These results indicated that basement membrane assembles with a significantly reduced rate in POMGnT1 deficiency. Reduced rate in assembly of the basement membrane may be unable to meet the demand of a rapidly enlarging cortical surface area, thus resulting in breaches.

Autocrine TGF-β1 Activation Mediates Confluence-Dependent Transcriptional Expression of Laminin-332 in MDCK Epithelial Cells.
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Laminin-332 (LN332) is a heterotrimeric protein (α3β3γ2) of the basement membrane and its abnormal expression has been correlated with numerous pathologies. In the kidney, LN332 is of particular importance because is transiently upregulated during epithelial regeneration after ischemia. However, the molecular signals that drive this transient expression are unknown. In Madin-Darby Canine Kidney (MDCK) epithelial cells, a cell culture model of the renal epithelium, LN332 is expressed only under subconfluent conditions when the cells lack completely intact cell-cell contacts and apical-basal polarity, and is turned off after reaching confluency. Expression of the α3 subunit, which likely controls secretion of the other LN332 subunits, is transcriptionally regulated, with high levels of mRNA in subconfluent cells and almost none in confluent cells. It has been reported that the promoter of the gene for α3 contains, among others, a TGF-β response element. Addition of exogenous active TGF-β1 to confluent MDCK monolayers is sufficient to induce transcription of the laminin α3 gene and LN332 protein expression via TGF-β type I receptor (TR1). Expression of LN332 in MDCK cells is likely an autocrine response because MDCK cells constitutively secrete endogenous latent TGF-β1, but only activate it under subconfluent conditions. TGF-β1 activation (but not secretion) is dependent on the αvβ3 integrin because a function-blocking anti-αvβ3 integrin antibody prevents TGF-β1 activation and reduces LN332 expression, suggesting an activation mechanism dependent on mechanical tension. In fully confluent and polarized cells, latent TGF-β1 is mainly secrete apically, while the TR1 and αvβ3 integrins are localized to the (baso)lateral domain, physically separating the ligand from the activation machinery and its receptor under conditions when there is no LN332 expression. Disruption of cell-cell contacts in such cultures is enough to stimulate LN332 expression, possibly mimicking the physiological response that occurs following injury to the renal epithelium.
Altogether, our data suggests a novel mechanism for LN332 transitory regulation in kidney epithelial cells following injuries that disrupt epithelial continuity.

463/B410
Intracellular Polarity Protein Par-1 Regulates Extracellular Laminin Assembly by Regulating the Dystroglycan Complex.
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Cell polarity depends on extrinsic spatial cues and intrinsic polarity proteins including PAR-aPKC proteins. In mammalian epithelial cells, cell-cell contacts provide spatial cues that activate the aPKC-PAR-3-PAR-6 complex to establish the landmark of the initial cellular asymmetry. PAR-1, a downstream target of the aPKC-PAR-3-PAR-6 complex, mediates further development of the apical and basolateral membrane domains. However, the relationships between the PAR-aPKC proteins and other extrinsic spatial cues provided by the extracellular matrix (ECM) remain unclear. Here, we show that PAR-1 is required for the assembly of extracellular laminin on the basal surface of mammalian cultured epithelial cell line, MDCK cells. PAR-1 is also indispensable for the basolateral localization of the dystroglycan (DG) complex, one of the laminin receptors essential for basement membrane formation, suggesting that PAR-1b affects extracellular laminin assembly by regulating this receptor complex. Consistently, loss of PAR-1 impairs the formation of a functional DG complex containing normally glycosylated alpha-DG and the spectrin-like actin-binding protein, utrophin. Furthermore, PAR-1 physically interacts with the DG complex through direct binding to the spectrin-like repeats of utrophin, and an alanine mutation of PAR-1-mediated phosphorylation sites on utrophin reduced the interaction between utrophin and dystroglycan. These results reveal the presence of a novel inside-out pathway in which an intracellular polarity protein regulates the ECM organization required for epithelial cell polarity and tissue morphogenesis.

464/B411
Circulating Fibrillin Fragments as Biomarkers for Marfan Syndrome.
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The Marfan Syndrome (MFS) is caused by mutations in the gene for fibrillin-1 (FBN1), the major component of microfibrils in the elastic matrix. The major manifestation of MFS is aortic aneurysm and dissection or rupture. Using a new Fbn1 mutant mouse model of MFS called GT-8 (a conditional eGFP-tagged knock-in truncation), we found that heterozygous GT-8 aorta showed increasing fragmentation of the elastic lamellae, beginning at 2 months of age, followed by aneurysm and dissection. Because the mutant fibrillin-1 is tagged with eGFP, we could determine—for the first time—that mutant fibrillin-1 is assembled into microfibrils in both the heterozygous and homozygous mutant mice. Studies of GT-8 mice demonstrated that the presence of mutant fibrillin-1 targets the microfibrils for proteolysis. Therefore, we hypothesized that microfibril fragmentation is a major mechanism of disease pathogenesis in MFS. To test this hypothesis, we developed a panel of sensitive sandwich ELISAs, utilizing monoclonal antibodies with defined epitopes, to quantitate circulating fragments of fibrillin-1 and fibrillin-2. Samples were collected from MFS (n=48) and control (n=25) volunteers. The results showed that in MFS samples the concentrations of the fibrillin-1 fragments, 15-201 and 15-26, were decreased compared to controls (p<0.0008). By contrast, circulating 72-143, a fibrillin-2 fragment, was increased in MFS relative to controls (p=0.04). To test whether this profile was specific to MFS, plasma samples from individuals with scleroderma as well as individuals with other forms of aortic aneurysm were assayed. Fibrillin-1 and fibrillin-2 concentrations were both decreased in scleroderma and were similar to the MFS signature in other individuals with aortic aneurysm. Strikingly, the relevant fragments that showed statistical significance (p<0.05) in MFS were different from those in the aneurysm samples. These results suggest that different proteases are
involved in the pathogenesis of MFS and other forms of aortic aneurysm. Defining these proteases may provide new targets for therapies.

**465/B412**

**Cytoplasmic Tails Delay Oligomerization of ZP2 and ZP3 Prior to Secretion and Formation of the Extracellular Zona Pellucida Surrounding Mouse Eggs.**

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The mouse zona pellucida (ZP1, ZP2, ZP3) forms an insoluble matrix surrounding ovulated eggs that is essential for fertilization and early development. Each component has a signal peptide, a conserved ‘zona’ domain, a C-terminal transmembrane domain and a short cytoplasmic tail. Only ZP2 and ZP3 are required to form a zona matrix, but little is known about the interaction of these two proteins necessary to assemble the extracellular zona pellucida. Objective: Here we adapt a fluorescent-based protein fragment complementation assay (BiFC) to detect ZP2-ZP3 interactions in the secretory pathway. Results: The N-terminal fragment (aa 1-156) of Venus was fused to ZP2 either just after the signal peptide (ZP2-NVenus) or just after the ‘zona’ domain (ZP2-CVenus). The C-terminal fragment of Venus (aa 157-239) was fused just after of the signal peptide of ZP3 (ZP3-NVenus). In addition, intact Venus and mCherry were fused just after the signal peptide of ZP2 and ZP3, respectively. To investigate the potential role of the highly conserved C-terminal tails, stop codons were introduced either just before or just after the transmembrane domain. ZP2/ZP3 proteins with the intact fluorescent and with the Venus fragments were co-expressed in mammalian cells and growing oocytes. The BiFC assay did not detect ZP2/ZP3 interactions within cells, but complementation was observed in secreted proteins with ZP2-NVenus (but not ZP2-CVenus) and ZP3-NVenus. However, each truncated zona protein (lacking a C-terminal tail by itself or in conjunction with the transmembrane domain) co-localized with the other and Venus complementation was detected inside cells. These studies were extended by microinjecting expression plasmids into growing oocytes and complementation was detected on the inner aspect of the zona matrix with ZP2-NVenus and ZP3-CVenus. More significantly, the BiFC assay did not detected ZP2/ZP3 interactions inside the oocyte unless they lacked their C-terminal cytoplasmic tails. Conclusions: Taken together, these data indicate that individually conserved C-terminal tails ensure that ZP2 and ZP3 traffic independently in the oocytes and that the two zona proteins interact via N-terminal domains only after they are secreted.

**Cell-Cell Interactions (466 – 490)**

**466/B413**

**Tracking CEACAM Dynamics and Interactions by Live Cell HOMO-FRET Microscopy.**

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The interactions between cells is critically important to a vast array of biological phenomena and events, ranging from cellular apoptosis to cancer and tumour development. The carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) represent a subset of the immunoglobulin superfamily involved in homophilic and heterophilic intercellular binding interactions that affect the control of cellular growth and differentiation. While CEA (CEACAM5) has been used as a marker for colon cancer progression for decades, inappropriate expression of CEACAM1 and CEACAM6 also leads to cellular transformation and tumourigenesis. While these effects are mediated by intercellular signals transduced by these receptors, the molecular basis for the interactions that govern intercellular binding and mediate CEACAM signaling are not known. In order to understand the biology of CEACAMs, we need to elucidate their distribution, expression and association with other proteins on the cell surface. These challenges represent...
the greatest impediments to the development of strategies that target CEACAMs for anti-tumour or other therapies. Using a combination of traditional molecular, biochemical and cellular analyses with our recently developed platform of functional molecular scale imaging based on the convergence of optical and scanning probe microscopies (SPM), coupled with single-molecule optical imaging and spectroscopy, we are examining whether CEACAM-dependent control of cellular growth and differentiation depends upon interactions that affect CEACAM orientation, structure and oligomerization state at the cell surface. Through a combination of live cell confocal and TIRF-based homo-FRET fluorescence anisotropy imaging, we have been able to calculate spatial maps of homo-FRET intensity that reflects differences in the monomer-dimer equilibrium at the cell surface as well as track CEACAM transport. Extensions of these approaches for tracking cell-cell interactions and the effect of small molecule and peptide-modulation of CEACAM equilibrium will be presented.

467/B414
SIRP-Alpha-CD47 System Functions as an Emergency Signal in Renal Glomerulus.
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Renal glomerulus consists with glomerular endothelial cells, podocytes and mesangial cells. Those cells cooperate with each other for the glomerular filtration. However, the intercellular signaling molecules among those cells have not so far been clarified. Perfusion of kidney with protamine sulfate (PS) induces foot process fusion and tyrosine phosphorylation of slit diaphragm molecules (ZO-1, nephrin and NEPH-1), suggesting that tyrosine phosphorylation is an indicator of the early event on the morphological changes in podocyte. Although src kinase is involved in this event, the molecules working for dephosphorylation are not fully determined. We found that SIRP-alpha is exclusively located in podocytes. SIRP-alpha is a type I transmembrane glycoprotein, which has three immunoglobulin-like domain in the extracellular region and two SH2 binding motif in the cytoplasm. This molecule functions as a scaffold to many proteins, especially SHP2-tyrosine phosphatase to the plasma membrane. SIRP-alpha is concentrated at the slit diaphragm region of podocyte. SIRP-alpha is heavily tyrosine-phosphorylated under the normal condition. on the other hand, PS-perfusion induced a dramatic decrease of tyrosine phosphorylation of SIRP-alpha. CD47, a ligand for SIRP-alpha, is also located in glomerulus, but its distribution is distinct from that of SIRP-alpha. CD47 is located along the plasma membrane of both mesangial and glomerular endothelial cells, but not podocytes. It is known that the cytoplasmic domain of SIPR-alpha is dephosphorylated, when CD47 is bound to the extracellular domain of SIRP-alpha. In normal glomerulus, mesangial cells can not attach directly to podocytes. However, when mesangial cells are injured by Thy1.1 monoclonal antibody injection, the phosphorylation of SIRP-alpha is dramatically decreased. The complex of SIRP-alpha and CD47 are incorporated in podocytes after mesangiolysis. The data suggest that SIRP-alpha-CD47 system plays a role in the cell-cell communication in diseased glomerulus.

468/B415
Membrane Signaling Proteins That Interact in Cis and Trans: Functional Implications of Competition.
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A large number of membrane proteins possess extracellular domains that interact with other membrane proteins and that also send important signals into cells, but it is also possible that such proteins interact in cis on the same cell in addition to trans, raising important questions of cis-vs-trans differences in signaling. Many cells express not only the ubiquitous 'Marker of Self' protein CD47 but also some level of its counter-receptor SIRPa (CD172), and we show that CD47-SIRPa interactions occur not only in trans in cell-cell adhesion, but also in cis, modulating downstream signals. With human macrophages in sparse culture, SIRPa is a phagocytosis inhibition receptor upon binding CD47 in trans (Tsai & Discher, JCB 2008), but SIRPa exhibits a a basal level of phospho-activations, which suggested that CD47 (which is expressed on macrophages) might also interact in cis. Activation is clearly increased with CD47-SIRPa interactions in trans, which
inhibits phagocytosis of any CD47-displaying target, whereas knockdown of CD47 on macrophages decreases the basal signaling, increases binding of soluble CD47, and enhances phagocytosis of opsonized targets above wild-type. With a series of knockdowns, the effective Kd for binding in trans is shown to depend linearly on cis CD47 on the macrophage, consistent with the simplest mathematical modeling for cis-vs-trans competition. Additionally, CHO cells engineered to express CD47-GFP bind more soluble SIRPα than CHO cells displaying both CD47 and SIRPα, although high affinity antibodies can out-compete cis interactions. Cis interactions might be unavoidable with this system and others studied (eg. MHC receptors, and Notch-Delta), but it is also possible that by lowering the threshold at which activation exceeds inhibition, cis interactions optimize discrimination in trans.

469/B416
Polymorphisms in Human SIRPα Effect in CD47 Interactions Leading to Incompatibility with Homologous Cells.
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CD47 (Integrin-Associated Protein) is a ubiquitous transmembrane protein that has been described to be a ‘marker of self.’ One of the determining interactions in phagocytic clearance of foreign cells has been demonstrated to be between CD47 and the transmembrane protein signal-regulatory protein alpha (SIRPα), which are present in professional phagocytes, including macrophages. CD47-SIRPα has been demonstrated to be species-specific leading to the phosphorylation of SIRPα. As a result, macrophages are able to distinguish between foreign and self cells. However, a high number of SIRPα polymorphisms exist in humans, which may have implications in homologous cell compatibility. In order to further characterize these polymorphisms, we used a CHO cell expression system to explore the binding affinities with the ligand CD47. Our results revealed that a subset of SIRPα showed a weaker binding to CD47, resulting in decreased SIRPα phosphorylation. The reduction in phosphorylation found in this subset of SIRPα polymorphisms may have an impact on stem cell therapies.

470/B417
IL-1RA Released by Corneal Epithelial Cells Induces Down-Regulation of MMP-2 in Cocultured Corneal Fibroblasts.
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The epithelial-mesenchymal interactions may play an important role in maintenance of corneal structure and function. And, cytokine-mediated signaling may thus occur either unidirectionally, from epithelial cells to fibroblasts or from fibroblasts to epithelial cells, or bidirectionally. We previously showed that corneal epithelial cells regulate the expression of N-cadherin and gap junction protein (Cx43) in corneal fibroblasts with the use of our coculture system. In this study, we investigate the physiological relevance of interactions between corneal epithelial cells and fibroblasts through the expression of metalloproteinases (MMPs) in epithelial-mesenchymal interaction in the cornea. The amounts of MMP-2 mRNA and protein in corneal fibroblasts were decreased by the presence of corneal epithelial cells. Corneal epithelial cells had no effect on the expression of the other metalloproteinases (MMPs) 1 in corneal fibroblasts. Furthermore, the amount of IL-1 receptor antagonist (IL-1RA) was increased by coculture in corneal epithelial cells number dependent manner. And depletion of IL-1RA in corneal epithelial cells by RNA interference largely abolished the effect of these cells on MMP-2 expression in fibroblasts. The down-regulation of MMP-2 expression in corneal fibroblasts by corneal epithelial cells was blocked by inhibitors of signaling by the mitogen-activated protein kinases ERK (PD98059), but not p38 MAPK (SB203580). Therefore, the presence of corneal epithelial cells down-regulated expression of MMP-2 in corneal fibroblasts, suggesting that corneal epithelial cells are important for maintenance of homeostasis for stable conditions mediated communication with corneal fibroblasts.
Desmosome Assembly and Maintenance in Migrating Cells.
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Desmosomes are prominent cell-cell adhesive junctions that anchor the intermediate filament cytoskeleton systems between adjacent cells in a tissue and provide the tissue the ability to withstand mechanical stress. The transmembrane core of the desmosome is comprised of cadherin family members, desmoglein and desmocollin. The cytoplasmic domain of the desmosomal cadherins associate with a number of desmosomal plaque proteins and in turn recruit the intermediate filament cytoskeleton to sites of cell-cell adhesion. The desmosomal plaque is comprised of two armadillo family members, plakoglobin and plakophilin and the plakin protein, desmoplakin. Cell-cell adhesion in a tissue is a dynamic process allowing individual cells the ability to change their location relative to neighboring cells. Tumor cells often display increased motility, lose adhesion to their neighbors and invade the surrounding stroma. In this study we examined the dynamics of desmosome assembly and junction localization in migrating oral SCC cell lines. In order to image desmosomes in live cells, desmosomal components (desmocollin 2a and plakophilin-3) were fused to GFP and stably expressed in UM-SCC-1 cells. Scratch assays were used to promote cell migration and desmosome assembly and maintenance was characterized. We observed initiation of new desmosome assembly near the leading edge of migrating cells and during cell migration the new desmosomal plaques are transported in a retrograde fashion. In addition, desmosomal plaques appear to accumulate some distance from the leading edge where their migration is decreased. Pre-treatment of the cells with drugs to disassemble the actin cytoskeleton altered the morphology of the cells however desmosome trafficking was unaffected. These studies begin to examine the complex process of coordination of desmosomal cell adhesion structures in migrating cells.

472/B419

Relavance of Antifeeze Protein and Connexin43 during Hypothermal Preservation.

Antifreeze proteins (AFPs) enable organisms to survive under freezing or sub-freezing conditions. Antifreeze protein inhibits the ice nucleation by adsorbing to the surfaces of ice nuclei. This leads to an increase of the ice nucleation barrier. These days, it has been used at many aspects which maintaining frozen food, cryopreservation of stem cell, blood, tissue and breed preservation of endangered animal. Cell-Cell Interaction is also important for multicellular organisms to survive. for example, It regulate cell attachment, metabolite migration, embryonic development and differentiation. Connexin43 is one of Cell-Cell Interaction constitution and most abundantly expressed in different cell types. We suggest that viability of cell is related with change of Connexin43. Antifreeze protein purified from antarctic yeast using his-tagged protein purification. Cell viability was analyzed on 4 centigrade by WST-8 assay after to cells added some protectants those are sugars, as antioxidant and energy source, UW solution and DMSO. Cell morphology was observed by fluorescein microscope in 600nm. In the final part of study, alteration of Connexin43 expression was identified by western blotting. As a result, Cell viability was increased on each cell. Also, those were visual different morphology between the control and samples added antifreeze protein. Alteration of Connexin43 expression demonstrates that cell-cell interaction affect to viability. This research fund was performed KOPRI PE09070.

473/B420

Endogenous Forces Exerted at E-Cadherin-Based Cell-Cell Contacts.
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Cadherins are the primary mediators of calcium-dependent cell-cell adhesion in all vertebrates and serve as the mechanical link between adjacent cells. However, an understanding of the regulation of forces sustained at cell-cell contacts mediated by cadherin is largely unknown. To
measure the forces exerted at E-cadherin interfaces in MDCK cells, we use high resolution traction force microscopy and confocal microscopy to image GFP-E-cadherin or GFP-vinculin, a marker of focal adhesions. Cells plated on matrices coated with collagen exert traction stresses via focal adhesions. In isolated cells, the traction stresses exerted on the underlying matrix are balanced across the cell. However, traction stresses measured in the underlying substrate are not balanced in cells interacting with a neighboring cell through a cadherin-based adhesion. The force imbalance, which reflects the force exerted at the cell-cell contact, is typically between 5-10 nN and directed away from the plane of the cell-cell contact. Calcium-switch measurements suggest that this force is distributed unevenly at the plane of cell-cell contact. These quantitative results of forces at cadherin-based contacts have important implications for the mechanical integrity of tissues and mechanotransduction at cell-cell contacts.

474/B421
Spatiotemporal Regulation of Epithelial Growth by the Quantitative Crosstalk between Cell-Cell Contact and EGF.
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Contact inhibition plays a key role in regulating the growth of epithelial tissue. While the mechanism of cadherin-mediated mechanisms of contact-inhibition is emerging, its quantitative aspects are still poorly understood. In particular, how cells quantitatively reconcile the opposing effects of cell-cell contact and soluble growth factors to make a “net decision” on cell cycle commitment remains unclear. Here, using quantitative analysis of single cells within multicellular clusters, we have demonstrated that contact is not a “master switch” that overrides epidermal growth factor (EGF). The mere presence of neighboring cells does not shut down epithelial cell proliferation. Rather, cell-cell contact inhibits proliferation only when the amount of soluble growth factor recedes below a critical threshold level. Furthermore, using micropatterned surfaces and molecular genetics approach, to modulate the amount of cell-cell interactions, we have demonstrated that this tipping point at which contact-inhibition is triggered is tunable, thereby providing means to manipulate the spatial dynamics of epithelial growth. This new quantitative model of contact-inhibition has direct implications for how tissue size may be determined and deregulated during development and tumor formation, respectively, and provides design principles for engineering epithelial tissue growth in applications such as tissue engineering.

475/B422
The Molecular Mechanisms on the Anti-Inflammatory Property of Thrombomodulin Lectin-Like Domain.
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Thrombomodulin (TM) is a type I transmembrane protein that exerts anti-coagulation and anti-inflammation. Previous studies show that the N-terminal lectin-like domain of TM (TM domain 1, TMD1) regulates inflammatory responses in models of sepsis, arthritis or ischemia. However, the mechanisms are still unclear. Our previous studies found that Lewis-y (Le-y) was a specific carbohydrate ligand that interacted with TMD1. Le-y belongs to one of the blood group antigens and recent studies show that the expression of Le-y correlates to malignancy and inflammation. Leukocyte recruitment is a complicated process in inflammation and carbohydrates play an important role in mediating cell rolling and adhesion. Therefore, we hypothesize that TMD1 reduces inflammation by regulating cell adhesion via interacting with Le-y. Our results showed that Le-y was expressed in various kinds of human endothelial cells and the expression level was upregulated upon tumor necrosis factor α (TNFα) stimulation. We generated FITC-conjugated recombinant TMD1 (rTMD1) protein to interact with human umbilical vein endothelial cells (HUVECs) and found that rTMD1 bound to HUVECs and the binding was increased under TNFα stimulation but decreased in the presence of Le-y carbohydrate. Next, we found that the adhesion of THP-1 monocytic cells to TNFα-activated HUVECs was inhibited by rTMD1 in static state and
shear flow condition (0.5 dyne/cm²). This inhibitory effect was blocked by preincubation of rTMD1 with Leα carbohydrate. We utilized TM knock-down THP-1 monocytic cells to investigate the function of TM on monocytes adhesion and the result showed that the adhesion was reduced upon TM knock-down. Our present results suggested that TM on monocytes may involve in leukocyte recruitment through binding to the carbohydrate ligand, Leα, on endothelial cells and these findings may explain the possible reason that TMD1 suppresses inflammatory events.

476/B423
The Role of the GEF Trio in ICAM-1-Mediated Leukocyte Transendothelial Migration.
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During inflammation, leukocytes transmigrate across the endothelium towards the site of injury. This process of extravasation requires integrin-mediated adhesion to endothelial adhesion receptors such as ICAM-1. Upon binding and clustering of ICAM-1, the small GTPases RhoA and Rac1 become activated, which induce endothelial cell shape changes to allow passage of transmigrating leukocytes. In addition, we demonstrated that another Rho family GTPase, RhoG, becomes activated after ICAM-1 engagement, controlling the cytoskeletal remodelling that are triggered downstream of ICAM-1 engagement. However, the signals leading to the activation of these small GTPases remain largely unclear. The guanine nucleotide exchange factor (GEF) Trio is able to activate RhoG, Rac1 and RhoA. We show here that Trio is expressed in endothelial cells and localized to F-actin stress fibers and cell borders. Moreover, the inflammatory stimuli TNFα, IL1β and LPS but not INFγ increase the mRNA and protein expression of Trio (~ 3-fold) in endothelial cells, but not in epithelial cells. A GFP-fusion protein of full-length Trio expressed in HeLa cells co-localized with ICAM-1-mCherry in lateral and apical membrane ruffles and is recruited to sites of ICAM-1 engagement. In addition, we show that full-length Trio is able to interact with a peptide encoding the intracellular tail of ICAM-1. Finally, using a GST-tagged nucleotide-free mutant of RhoG (G15A), we demonstrate that ICAM-1 clustering activates endogenous Trio in endothelial cells. Current studies are focussed on the role of endothelial Trio in the adhesion to and migration across the endothelium of leukocytes. Thus, ICAM-1-mediated activation the GEF Trio may a key event in ICAM-1-mediated endothelial signalling through a set of Rho-like GTPases, which is critical for the engagement of ICAM-1 as well as for efficient leukocyte transendothelial migration.

477/B424
Dynamics of Rac1 GTPase Activity at De Novo Cell Adhesions.
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The formation of cell-cell contacts involves the spatiotemporal regulation of protein complexes, including the trafficking and engagement of cadherins and reorganization of the actin cytoskeleton by Rho GTPases. Previous work identified the role of E-cadherin engagement in establishing localized zones of Rac and Rho GTPase activities during initial cell-cell contact. How initial cell-cell adhesion creates waves of Rac1 activation and inactivation as cell contacts expand remains unknown. In this study, we examined the immediate effects of cell engagement on Rac1 activity levels. Although published biochemical studies have implicated E-cadherin in the local activation of Rac1 at initial cell-cell contacts, our preliminary studies demonstrate that Rac1 activity upon cell-cell contact does not depend solely on E-cadherin engagement. We show that when cells make contact with their neighbors in a 3-D cell aggregate, the initiation of E-cadherin engagement results in a very rapid decrease in Rac1 activity. However, when cells spread in 2-D on a collagen surface make contact with neighboring cells, we find that there is a transient increase in Rac1 activity followed by a rapid decrease in Rac1 activity. These data suggest that cadherins and integrins cooperate to regulate Rac1 activity during initial cell-cell adhesion, and that the waves of Rac1 localization and activity observed in live cells depends on multiple proteins and signaling pathways. Future work aims to decipher the guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) involved in regulating Rac1 activity during
immediate cell-cell adhesion, and the signaling pathways involved in the spatiotemporal regulation of these protein complexes at cell-cell contacts.

478/B425

Roles of Cdc42 in Yeast Cell Fusion.

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Haploid yeast cells mate to produce a diploid zygote. Mating entails cell fusion, requiring the localized breakdown of the intervening cell walls and fusion of the plasma membranes. Proteins required for efficient cell fusion include Rvs161p, an amphiphysin, and its binding partner, Fus2p. Fus2p contains a Dbl homology (DH) domain, suggesting that it acts as a GTP-exchange factor for a Rho-GTPase in yeast, and the DH domain is necessary for fusion. Three lines of evidence identified Cdc42p as the best candidate for the Rho protein regulated by Fus2p. First, high copy expression of BEM1, encoding a scaffolding protein for Cdc42p, suppressed a fus2 mutant. Second, a previous high-throughput 2-hybrid screen reported an interaction between them. Third, Cdc42p is the only Rho protein in yeast known to be required for mating, although previous work suggested that it is required for actin organization and polarization. To identify a specific role for Cdc42p in cell fusion, separate from actin polarization, we screened a collection of alanine-scanning cdc42 mutants. Two alleles were identified, cdc42-137 and cdc42-138, due to their reduced mating with fus1 fus2 mutants, but not wild-type cells. Microscopy showed that both mutations alone caused cell fusion defects. The defects were not due to reduced pheromone-dependent signaling, because a STE20-ACR1B mutation, which isolates Cdc42p from the pheromone response pathway, did not abrogate the effect of cdc42-138. In contrast to other cdc42 alleles, the cdc42-138 defect was not a secondary consequence of inefficient polarization; cdc42-138 cells formed shmoos with polarized actin (and other proteins required for cell fusion) as efficiently as wild-type. By electron microscopy, the morphology of cdc42-138 zygotes was virtually identical to fus2 and rvs161 zygotes. Finally, double mutant analysis demonstrated that fus2 and cdc42-138 act in the same pathway. Thus, Cdc42p has several roles in yeast cell fusion, regulating both actin polarization and pheromone signaling, as well as acting together with Fus2p late in the pathway. A function for Cdc42p is consistent with a conserved role for the Rho/Rac family of G-proteins in eukaryotic cell fusion.

479/B426

Spatial and Mechanical Aspects of EphA2 Signaling.

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The EphA2 receptor tyrosine kinase regulates intercellular adhesion and motility via interactions with its activating ligand ephrin-A1, expressed on the surface of apposing cells. Abnormal EphA2 levels are an established biomarker of tumorigenicity in neoplastic tissue, and increasing its expression in noninvasive cells has been shown to confer malignancy. However, the precise relationship between EphA2 activity and the pathological program of increased motility and decreased adhesion that characterizes invasive behavior in individual cells remains unclear. Here, we reconstitute the native intercellular signaling geometry using a hybrid junction between live human breast cancer cells expressing EphA2 and a supported membrane functionalized with ephrin-A1. After cells contact the membrane for 1 hour, we observe spatial reorganization of EphA2-ephrin-A1 complexes on multiple lengthscales as well as changes in cortical F-actin organization and recruitment of membrane metalloprotease ADAM10. Mechanical restriction of EphA2-ephrinA1 complex movement alters the cellular response to ephrin-A1. Here we find that abolishing actomyosin contractility with ROCK-inhibiting drugs prevents largescale EphA2 reorganization in a similar way. Both biochemical and mechanical methods of preventing EphA2 reorganization lead to the same result: we no longer observe either F-actin reorganization nor the recruitment of ADAM10 to EphA2 clusters. To further probe the radial polarization phenomenon, we have investigated a panel of membrane-associated effector proteins with functions associated with motility and membrane targeting to determine how they are affected by polarization. With these observations, we hope to elucidate the link between EphA2 and the irregularities in cytoskeletal morphology and cell behavior that accompany tumor invasion.
480/B427
Role of Spatial Organization in Receptor Function: Eph-Ephrin Signaling in Breast Cancer.

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Activation of the EphA2 receptor tyrosine kinase by its natively membrane-bound ligand, ephrin-A1, plays an important role in breast cancer biology. In Vivo receptor-ligand binding is followed by EphA2-ephrin A1 complex formation, activation of the receptor, and ultimately receptor endocytosis and degradation. These events are all recapitulated when EphA2-expressing human breast cancer cells are cultured on a supported lipid bilayer presenting laterally mobile, membrane-tethered ephrin-A1. However ligand-induced receptor clustering and activity is altered when the ligand is presented in solution or bound to solid surfaces. Real-time fluorescence imaging, immunostaining, and protein expression analyses all show that membrane-presented ligand induces a global receptor spatial reorganization phenotype. Moreover, this phenotype correlates with disease characteristics and is linked to the expression of a subset of proteomic and genomic cancer biomarkers. Using nanopatterned substrates to impose geometrically defined barriers to lateral mobility, it is possible to restrict and guide the assembly of these patterns in living cells. We refer to this technique as a spatial mutation. This mechanically-induced EphA2 reorganization alters the cellular response to ephrin-A1 as observed by changes in cytoskeleton morphology and recruitment of the protease ADAM10. Our results indicate that mechanical restriction of EphA2 pattern formation can alter the outcome of its signaling pathway, which may also have consequences for certain aspects of cancer metastasis.

481/B428
Basigin Gene Products Form a Novel Adhesion System between Photoreceptors and Müller Glia in the Vertebrate Neural Retina.

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In the neural retina, photoreceptor neurons and Müller glia are in intimate contact with one another. It is now known that Basigin gene products, members of the immunoglobulin superfamily, form a novel adhesion system that is crucial for photoreceptor function. Mice in which Basigin gene products have been deleted are blind from birth since photoreceptor neurons have limited responses to light in those animals. There are two Basigin gene products expressed in the neural retina. Basigin is found on the surface of Müller cells, whereas Basigin-2 is found on the surface of photoreceptors. The two polypeptides are identical in amino acid sequence, with an additional 116 amino acids in Basigin-2 that form an immunoglobulin loop at the amino (N)-terminus of the molecule. The purpose of this study was to characterize the interaction between Basigin gene products of the neural retina. Binding assays were performed using an ELISA protocol in which endogenous mouse Basigin, from both retina and kidney, was probed with recombinant Basigin-2-specific loop. The Basigin-2 loop was then truncated via molecular mechanisms to determine the amino acids used to bind Basigin. A BLAST search was also employed to identify proteins homologous to the Basigin-2-specific amino acid sequence. Basigin-2 does bind Basigin via its N-terminal immunoglobulin loop. Assays using Basigin from mouse kidney confirmed that the interaction is heterophilic, as Basigin-2 is not expressed in that tissue. The affinity of the interaction is rather weak, with a KD of approximately 2 microM. Amino acids within the N-half of the Basigin-2 loop bind to Basigin. This region has high amino acid sequence identity (66%) to L1 neural cell adhesion molecule, a known binding partner for Basigin in brain. It is thought that the Basigin gene products associate in the vertebrate retina as part of a monocarboxylate shuttle complex in which monocarboxylates (lactate, pyruvate) are transported from Müller cells to photoreceptors to fuel oxidative phosphorylation in the neurons. Absence of this complex likely leads to reduced energy output in photoreceptor neurons and is thought to be the molecular mechanism underlying blindness in Basigin null mice.
A Central Role of Radial Glial Stem Cells in Orchestrating Neural as Well as Vascular Development in the Cerebral Cortex.

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Cerebral corticogenesis involves the production, migration, and differentiation of multiple neural and non-neural cell lineages born inside and outside the cortex. Radial glia are well known for their roles in regulating the production and migration of neurons born in the cortical ventricular zone. Here we show that radial glia guide the migration and localization of Cajal-Reztius (CR) cells born outside the cortex as well as regulate the process of cortical vascular development. To determine the role of radial glia in CR cell migration, we took advantage of a β1 integrin conditional mutation to perturb cortical radial glial scaffold. We found that this resulted in the displacement of CR cells from the cortical marginal zone into deeper layers. The displacements closely correlated with local retraction of radial glial endfeet from the pia. They also took place before basement membrane defects and did not result from cell autonomous requirement for β1 integrin. Further, perturbation of radial glial scaffold by interfering with cell division resulted in a similar phenotype of local radial glial loss and CR cell displacement. These results thus reveal a crucial role of radial glia in coordinating the integration of CR cells from their extracortical origins into the cortex. To determine the role of radial glia in cortical vascular development, we employed an orc3 conditional allele to block radial glial division. We found that radial glial depletion resulted in defective blood vessel formation and cerebral hemorrhage at perinatal stages. Cortical blood vessels developed normally at early stages but stopped growing and regressed following radial glial depletion. Increased endothelial cell death was observed in the mutant cortex, concomitant with defective signaling in several pathways key to brain angiogenesis. These results thus identify a novel role of radial glia in coordinating cortical angiogenesis. Together, our results suggest that radial glia, through coordinating multiple neural and non-neural processes of cortical development, play a central role in orchestrating the program of corticogenesis.

Na,K-ATPase ß-Subunit Mediated Cell-Cell Adhesion Is Necessary for Epithelial Lumen Formation in Mammalian Cells.

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Na,K-ATPase is a hetero-oligomer of an α- and a β-subunit. The α-subunit (Na,K-α) has the catalytic function of transporting two potassium ions into and three sodium ions out of the cell. The Na,K-ATPase ß-subunit (Na,K-ß) is involved in the regulation of the ion transport activity. We have shown that the Na,K-ß1 isoform has cell-cell adhesion function and is localized to the apical junctional complex in polarized epithelial cells. In cancer Na,K-ß1 expression is reduced and replenishing this protein in carcinoma cells induced epithelial polarity. However, the mechanism by which Na,K-ß1 induces epithelial polarity is not understood. We recently identified two distinct conserved motifs on the Na,K-ß1 transmembrane domain that mediate protein-protein interactions: A glycine zipper motif involved in the homo-oligomerization of Na,K-ß1 and a heptad repeat that is involved in the hetero-oligomeric interaction of Na,K-ß1 with Na,K-α. Point mutations in the heptad repeat motif reduced Na,K-ß1 binding to Na,K-α and Na,K-ATPase activity. The Na,K-ß1 transmembrane domain homo-oligomerizes in biological membranes and mutation of the glycine zipper motif affected oligomerization and cell-cell adhesion function (Barwe et al., J. Mol. Biol., 365; 706-14; 2007). Recent studies in Drosophila showed that Na,K-ATPase is involved in epithelial polarity and forms a complex with a novel group of polarity proteins,Yurt, Coracle, and Neurexin IV (Laprise et al., Nature, 459; 1141-5; 2009). MDCK cells when grown in collagen form cysts with a lumen and have been used as a model to identify mechanisms involved in the formation of apical and basolateral domains during epithelial polarization. We now provide
evidence that RNAi mediated knockdown of Na,K-β1 prevents the lumen formation in MDCK cysts. This phenotype could be rescued with wildtype Na,K-β1 and heptad repeat mutants but not with glycine zipper mutants that abrogate the Na,K-β1 mediated cell-cell adhesion function. These studies demonstrate that the Na,K-β1 cell-cell adhesion is necessary for epithelial lumen formation in mammalian cells.

484/B431

**Inhibition of Bicc1 Impairs Tubulomorphogenesis of Cultured Imcd Cells by Disrupting E-Cadherin-Based Cell-Cell Adhesion.**

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Bicaudal-C (Bic-C) gene was originally discovered in Drosophila melanogaster. The gene product Bic-C is assumed to serve as an RNA-binding molecule. Loss of mouse Bic-C homologue (Bicc1) results in phenotypes mimicking human hereditary polycystic kidney disease (PKD). To characterize the unknown cellular function of Bicc1 in mammalian system, we established Bicc1-silenced cell lines from mouse inner medullary collecting ducts (IMCD) by short hairpin RNA (shRNA). 95% of Bicc1-silenced IMCD cells failed to establish tubulogenesis, compared to less than 10% of wildtype. To determine what contribute to this defect, we systematically examined characteristics of Bicc1-silenced IMCD cells. The junctional staining of E-cadherin was markedly indistinguishable and more cytosolic. The cortical actin distribution, cytoskeleton organization and cell shape was abnormal, and stress fibers were thick and abundant. Treated with apoptosis inducer ionomycine, more than 25% of Bicc1-silenced IMCD cells underwent programmed cell death, much higher than wild-type and control cells (5-10%). However, the proliferation rate of Bicc1-silenced IMCD cells was lower than wild-type and control cells, suggesting Bicc1 might exert a “gain of function” regulation during tubulogenesis. In addition, we also examined the ciliogenesis of different cell lines. Approximately 83% of wildtype and control IMCD cells showed ciliary staining, while less than 20% of Bicc1-silenced IMCD cells were stained. To determine the subcellular localization of Biccl, we co-stained IMCD cells with anti-Biccl polyclonal antibody and anti-acetylated α-tubulin antibody. We found that Biccl predominantly displays a cytosol distribution. Hela cells were transiently co-transfected with expression constructs of Biccl and Argonaute—a protein localized to P-bodies, the result showed that Biccl colocalized with Argonaute, which indicated that Biccl may serve as a P-body associated protein. The observations of aberrant cellular behaviors in Bicc1-silenced IMCD cells revealed functions of Biccl in renal epithelial cells and may benefit an insight into the pathogenesis of PKD.

485/B432

**Identification of a Cell Adhesion Molecule LagB as the Heterophilic Receptor of LagC in Dictyostelium Discoideum.**

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Dictyostelium discoideum grows as unicellular amoeboid cells which aggregate into a multicellular organism upon starvation. Multicellularity is maintained by the expression of several cell adhesion systems. The cell adhesion molecule LagC/gp150 accumulates rapidly in the early mound stage and plays crucial roles in the regulation of cell differentiation and cell sorting in the post-aggregation stages. LagC/gp150 is known to mediate cell-cell adhesion via heterophilic interaction with a membrane protein in a Ca2+/Mg2+-independent manner. Using recombinant His6-LagC to probe far western blots, we have identified a 130 kDa ConA-binding plasma membrane protein as the LagC heterophilic receptor. Results from LC/MS/MS mass spectrometry indicate that the 130 kDa glycoprotein is the product of the lagB gene. Interestingly, both lagB and lagC genes are located adjacent to each other in the opposite direction on chromosome 3. They share the same promoter and display similar expression profiles. Recombinant proteins of LagC and LagB can pull down their respective endogenous binding partner from cell lysate derived from post-aggregation stage cells but not vegetative cells. Binding of recombinant His6-LagB to live cells followed by anti-His antibody leads to the co-clustering of His6-LagB and LagC
on the cell membrane, demonstrating trans interactions between these two proteins. Cells constitutively expressing LagB and LagC can aggregate together during vegetative growth. Additionally, antibodies raised against LagB inhibit EDTA-resistant cell-cell adhesion in the post-aggregation stages. Development is impaired when cells are incubated with the anti-LagB antibody, resulting in a substantial reduction in fruiting body formation and spore yield. Our results suggest that LagB and LagC interactions are critical for Dictyostelium morphogenesis and spore cell differentiation in the post-aggregation stages of development. (Supported by Canadian Institutes of Health Research)

486/B433

Trans-Interactions between the IPT/TIG Domain of Two Cell Surface Glycoproteins Mediate Stage-Specific Cell-Cell Adhesion during Dictyostelium Development.

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IPT/TIG domains are found in cell surface receptors such as Met and Ron as well as intracellular transcription factors involved in DNA binding. Membrane proteins of the IPT/TIG domain subfamily share several functional features including cell dissociation, motility, and invasive activities. What are the functions of these IPT/TIG domains? Our studies have led to the identification of a pair of IPT/TIG-containing membrane glycoproteins, LagB and LagC, which interact heterophilically to mediate cell-cell adhesion in the post-aggregation stage of Dictyostelium development. LagB contains one IPT/TIG domain while LagC contains two IPT/TIG domains. Binding studies using various recombinant protein fragments have led to the identification of the LagC IPT/TIG-2 domain as the binding site for LagB. Peptide competition studies have further mapped the site to the sequence between Ile-336 and Val-360. Using recombinant fragments of LagB to probe far western blots, we have localized the heterophilic binding site of LagB to the C-terminal one-third of the extracellular region of LagB. Since this region also contains an IPT/TIG domain, a His-tagged protein containing the LagB IPT/TIG domain was used to probe far western blots. Significantly, the data show that the LagB IPT/TIG domain binds with the IPT/TIG-2 domain of LagC. Peptides corresponding to the LagC TIG-2 domain inhibit this interaction. These peptides also inhibit cell adhesion in cell-to-cell binding assays. Taken together, our results show for the first time that interactions between the IPT/TIG domains of two different membrane glycoproteins mediate stage-specific adhesion among cells during development. (Supported by Canadian Institutes of Health Research)

487/B434

Role of DdCAD-1-Mediated Cell Adhesion in Dictyostelium Pattern Formation.

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DdCAD-1 is a Ca^{2+}-dependent cell adhesion molecule encoded by the cadA gene, which is expressed by the social amoeba Dictyostelium discoideum at the onset of development and present throughout development. The NMR solution structure of DdCAD-1 reveals two domains with β-sandwich architecture. DdCAD-1 is synthesized as a soluble protein and then transported to the plasma membrane through an unconventional transport pathway via contractile vacuoles. To investigate the biological roles of DdCAD-1, DdCAD-1-GFP was expressed in cadA-null cells. Both temporal and spatial regulation of DdCAD-1 expression became evident. At the slug migration and early culmination stages, cells in the anterior region contained higher levels of DdCAD-1. In order to test whether cells expressing higher levels of DdCAD-1 would sort to the anterior region, chimera experiments were performed by mixing DdCAD-1-GFP expressing cells and cadA-null cells. DdCAD-1-GFP expressing cells sorted out from the null cells and became localized to the anterior region. Notably, at the final stage of the development cadA-null cells were able to form viable spores and behaved like cheaters. Quantitative analysis of spores in these chimera structures indicated that cadA gene acted as a single gene green beard similar to the csA gene in Dictyostelium, which also codes for a homophilic cell adhesion molecule. Further analysis showed that DdCAD-1-GFP was present in cell-cell contacts among cells in the anterior
region but not in the posterior region. Cells expressing mutant DdCAD-1 that failed to reach the cell surface also exhibited defects in cell sorting and behaved like cadA-null cells in chimeras. Taken together, these observations suggest that differential cell adhesiveness contributed by DdCAD-1 in the post-aggregation stages of development is responsible for the cell sorting phenomenon, which underlie the mechanism of green beard behaviour of the cadA gene. (Supported by Canadian Institutes of Health Research)

488/B435
Quantitative Evaluation of Characterized Alpha and Beta Glycosidase Effects on Sea Urchin Embryo Cellular Interactions.
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Using the NIH designated sea urchin embryo model, we are studying the molecular basis of a set of cellular interactions that have interested investigators for over a century, archenteron organization/elongation/attachment to the blastocoel roof. Here we evaluate the effects of four commercially available glycosidases: alpha and beta amylase and alpha and beta glucosidase, that we independently characterized, on embryo development. In quantitative dose-response experiments we show an I-50 of 10-30 units/ml, in artificial seawater or low calcium artificial seawater, of beta amylase and alpha glucosidase in inhibiting archenteron organization/elongation/attachment to the blastocoel roof in living 48 hr Lytechinus pictus gastrula embryos using a microplate assay that allows quantitative assessment of embryo morphological characteristics. All concentrations tested (up to 5000 units/ml) of alpha amylase and beta glucosidase had no significant effects on archenteron morphology. All experiments involved assessing 3600-5600 embryos in at least 144 replicate wells for each enzyme. The differences in archenteron morphology compared with controls were significant to p values of less than 0.05, while any differences observed with the ineffective enzymes had p values of greater than 0.05, using two-tailed t-tests. All enzymes used were tested for their activity on known substrates, and found to be active, and specific unit activities were established. Product inhibition studies suggested that the effective enzymes acted by their specific glycosidase activities and polyacrylamide gel electrophoresis suggested that there was no detectible protease activity in the enzyme samples. The results suggest that glycans are involved in these cellular interactions and we propose possible conformations of these glycans (Supported by NIH NIGMS SCORE (S0648680), MARC, RISE, the Joseph Drown Foundation and the Sidney Stern Memorial Trust).

489/B436
Cell Differentiation and Colony Formation in the Choanoflagellate Proterospongia sp.
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Choanoflagellates, the closest known relatives of animals, promise to help reveal the origins of animal cell-cell interactions and cell differentiation. Here we examine Proterospongia sp., a choanoflagellate that transitions between single-celled and colonial forms under laboratory conditions. We show that Proterospongia undergoes cell differentiation during the transition from substrate-attached cells to free-swimming colonies, marked by a glycosylation change at the plasma membrane that is revealed by specific labeling with the lectin wheat germ agglutinin (WGA). Unlike substrate-attached cells, differentiated cells in the water column remain attached to each other following cell division, with fine cytoplasmic bridges that connect the cell bodies to form chains. Addition of Algoriphagus bacteria to Proterospongia cultures results in the development of colonies with a rosette morphology, in which cells attached by cytoplasmic bridges are arranged spherically around, and extend filopodia into, a central core. Substrate-attached cells secrete goblet-shaped thecae that hold the cell body in a cup at the apex of a fine stalk. We find that cultures fed on Algoriphagus bacteria have thecae with reduced stalk length, revealing a potential trade-off between theca formation and colony development. Future studies
of theca morphogenesis and cell attachment within colonies promise to illuminate the potential adaptive significance of colony formation, and the molecular mechanisms of cell-cell interactions in choanoflagellates.

490/B437

**Bioastronautics: The Effect of Microgravity on Human Stem Cells.**

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A new field called bioastronautics has emerged in life sciences to ensure the health and safety of humans during space travel by addressing medical issues encountered, such as the absence of gravity. The aim of the present study was to employ a global approach to examine the effect of microgravity on stem cells. This was achieved using a NASA rotating-wall vessel bioreactor to simulate microgravity. The effect of microgravity was analysed on an embryonic stem cell line by utilising two-dimensional gel electrophoresis and Fourier Transform Mass Spectrometry to generate a comparative expression profile and to isolate and identify proteins of cells grown under microgravity. In addition, microarrays were used to generate a comparative genomic expression profile which was compared to proteomic analyses. Scanning Electron Microscopy (SEM) was also used to examine morphological adaptations to microgravity. Results demonstrated that 75% of proteins identified in the conditioned media from cells grown in microgravity were not found in control samples. Proteomic analysis demonstrated alterations in the expression of key proteins from cells grown under microgravity. As the functional unit of a cell are proteins, this suggests that microgravity has a significant impact on cell function. Similar trends were also observed at the genomic level whereby results showed considerable modifications to the genomic expression profile. SEM revealed that cells grown under microgravity exhibited an altered extracellular matrix that is thought to be due to growth under microgravity conditions. This is the first study conducted that has investigated the effect of microgravity on this embryonic stem cell line using both a proteomic and a genomic approach, demonstrating a significant alteration in human cell function as a result of growth in microgravity. This research is critical to space science and cell biology due to its vast potential to decipher the effect of microgravity on humans at a cellular level as well as regeneration of tissues in the human body and to use these alterations to solve ground based medical problems.

**Tight Junctions (491 – 499)**

491/B438

**Overexpression of Claudin-3 in a Breast Cancer Cell Line.**

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Claudin-3, one of the 24 identified members of the claudin family, is an integral membrane protein that helps form vertebrate tight junctions (TJs). The claudin proteins interact with other TJ proteins to determine cell polarity and specific ion diffusion through the paracellular pathway. Recently, several investigations have suggested that claudin proteins might be involved in cell signaling pathways. In this study, we showed that the breast cancer cell line MCF-7 markedly overexpresses claudin-3 protein, but can form TJs with a relatively high transepithelial resistance (TER). We used small-interference RNA (siRNA) to suppress claudin-3 protein expression to that of near normal levels in the MCF-7 cells over a period of 7 days. We observed a decrease in growth rate and TER in these transfected cells (normal claudin-3 levels) compared to nontransfected MCF-7 cells (high claudin-3 levels). Using MCF-7 cells that express high claudin levels, we have shown that the claudin-3 protein is readily detected in all cell fractions, the cytosolic, membranous, nuclear, and cytoskeletal fractions. We are currently investigating the cellular localization of claudin-3 protein in MCF-7 cells transfected with claudin-3 siRNA. The unusual localization of an integral membrane protein may further indicate a role for claudin
proteins in cell signaling pathways. The cellular localization of the claudin-3 protein and functional consequences may shed light upon the deregulation of TJ function in

**492/B439**
**Structural and Functional Aspects of Claudins Forming Tight Junctions in the Brain.**
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The structure of claudin-1, -2, -3, -5, and -12 expressed at the tight junction (TJ) in the brain is unknown. Claudin-5 tightens the cleft between endothelial cells forming the blood-brain barrier, and prevents paracellular permeation of small molecules. Claudin-2 forms paracellular cation pores between epithelial cells of the blood-cerebrospinal fluid barrier. The cerebral function of the other claudins remains unknown. To clarify the molecular interaction mechanism we investigated the oligomerization and strand formation of claudins using single amino acid exchanges. Mutants of claudin-5 and other claudins transfected into TJ-free cells demonstrated that the second extracellular loop (ECL2) is involved in the strand formation via trans-interaction (between opposing cells), but not via polymerization along the plasma membrane of one cell (cis-interaction). Combining the results of mutagenesis, live-cell imaging, electron microscopy, tightness studies and molecular modelling led to an antiparallel homodimer model of the ECL2. This may explain how two claudins hold onto each other and constrict the paracellular space. The interface includes an aromatic binding core and hydrophilic residues. On the basis of our data, we establish a novel molecular concept for TJ formation considering homo- and heterologous claudin-claudin interactions.

**493/B440**
**Inhibition of Cell Migration by Claudin-7 Is Mediated through p42/44 MAPK Signaling Pathway.**
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Lung cancer is the leading cause of cancer deaths worldwide and responsible for 1.3 million deaths annually with a very poor overall 5-year survival rate. Recently, we found that claudin-7, a tight junction integral membrane protein, was either down-regulated or its distribution was disrupted in human lung cancer. Using tissue microarray (TMA) analysis, we showed that claudin-7 was distributed to cell-cell junction in normal lung epithelia, whereas its localization in lung cancer epithelia was either discontinuous, disperse in cytoplasm, or it was completely absent in many areas. To investigate claudin-7 function in lung cancer cells, we transfected claudin-7 cDNA into NCI-H1299, a human lung carcinoma cell line that has no detectable claudin-7 expression. Cells transfected with vector alone were used as a negative control. Our results showed that without any treatment, claudin-7 expression did not affect the expression level of p42/44 MAPK (ERK1/2) and cell migration using wound-healing assay and imaging analysis. However, when treated with hepatocyte growth factor (HGF) that activates p42/44 MAPK pathway, claudin-7 expressing cells showed a decreased response to HGF in that they were less motile and formed less foot processes compared to the cells transfected with vector alone. In addition, western blot analyses revealed that cells expressing claudin-7 decreased the upregulation of phospho-ERK1/2 expression upon HGF treatment in a time-dependent fashion. Cell migration and expression of phospho-ERK1/2 were completely inhibited by MEK1 inhibitor, PD98059. Taken together, our results indicate that claudin-7 inhibits cell migration through p42/44 MAPK signaling pathway in NCI-H1299 human lung cancer cells.

**494/B441**
**Hormone Ouabain Modulates the Tight Junction.**
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Given that toxic levels of ouabain (more than 300 nM) disrupt several types of cell contacts, such as tight junctions (TJ), gap and adhesion to neighbors and substrate, in a previous work we suggested that the physiological role of ouabain may consist in the modulation of cell adhesion. Exploring further this possibility we now demonstrate that, at concentrations as low as 10 nM, ouabain does not detach the cells, triggers apoptosis, or abolishes the barrier function of the TJ, which are typical phenomena observed at toxic levels. Nevertheless it enhances the degree of sealing of the TJ, and specifically decreases the unidirectional flux of 3 kDa Dextran through the paracellular permeation route. These effects correlate with modifications of the amount of expression and pattern of distribution of claudins -1, -2, -3, -4 and -8 but not of occludin. Changes of a given type of claudin follow its own kinetics, which is not observed with the other claudin species tested. We focus on claudin-1 to further study signals and mechanisms, and find that ouabain enhances cln-1 expression by increasing its transcription, and the amounts of its mRNA. These responses are orchestrated by a complex cascade of signals triggered by ouabain and includes c-Src and the ERK1/2 complex. In summary, this work supports the view that hormone ouabain modulates cell-cell contacts in a highly specific way.

495/B442

**Ral GTPases Differentially Regulate Tight Junction Assembly and Function.**

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Tight junctions (TJs) serve an essential barrier function in epithelial and endothelial cells that is crucial to human health, but a detailed mechanistic understanding of how TJs form is lacking. TJ proteins must assemble at the junction between apical and lateral membrane domains to function as a permeability barrier, but factors responsible for targeting transport vesicles to developing TJs have not been identified. The Exocyst, under control of Ral GTPases, is known to regulate basolateral trafficking. Here, we have investigated the role of Ral GTPases in TJ assembly. During establishment of TJs in MDCK cells, trans-epithelial resistance (TER) displays a brief overshoot 4-5 times steady state levels. While this observation is well documented in the literature, its molecular basis is unknown. Silencing expression of either RalA or RalB drastically affects TJ formation and composition, although the direct consequences of each knockdown are radically different. Knockdown of RalA results in loss of the TER overshoot, while knockdown of RalB greatly exaggerates the overshoot. In both cases, TER reaches steady state levels comparable to control cells within 24 hours. TER profiles observed in knockdown cells are restored to normal upon expression of hairpin resistant RalA or RalB isoforms. Furthermore, data indicates that RalA exerts its effects on TJ formation through interactions with the Exocyst. Cells expressing inducible RalA mutants incapable of binding Sec5 or Exo84 do not display a TER overshoot. We postulate that differences in TER overshoot result from different rates of trafficking or exocytosis of TJ proteins. Consistent with this idea, composition of developing TJs is altered in Ral knockdown cells; incorporation of certain claudins into insoluble TJ complexes is either increased or decreased relative to control cells. Our working model is that RalA facilitates trafficking of TJ constituents to the plasma membrane, where RalB then ensures correct spatio-temporal fusion of transport vesicles and/or incorporation of these components into developing TJs. Future work will increase our understanding of how these GTPases, in collaboration with the Exocyst, contribute to TJ formation.

496/B443

**NFκB Inhibitors Disrupt Alveolar Epithelial Tight Junctions.**

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Tight junctions are required for the air/liquid barrier function of pulmonary alveolar epithelial cells. Tight junctions consist of several classes of transmembrane and scaffold proteins, including claudins, occludin, junction adhesion molecules (JAMs), zonula occludens (ZO)-1 and ZO-2 which act in concert to maintain a selective paracellular permeability barrier. We used inhibitors of
the NFκB signaling pathway to determine whether this transcription factor has a role in regulating alveolar epithelial tight junctions. Primary rat type II alveolar epithelial cells were isolated and cultured on Transwell permeable supports coated with collagen for five days to generate a model type I cell monolayer. Model type I cells formed a tight barrier as measured by transepithelial resistance (TER) values in the range of 500-700 Ohm x cm². Treatment of the monolayers overnight with either Bay-11 or BMS-345541 resulted in a dose-dependent decrease in TER at concentrations that did not affect cell viability. By immunoblot, there was little effect on the total amount of cell-associated claudin-4, -5, -7, -18, occludin, JAM-A, ZO-1 or ZO-2. However, the localization of these proteins at the plasma membrane was disrupted as assessed by immunofluorescence microscopy. There were several areas of cell-cell contact which lacked tight junctions and there appeared to be an increase in intracellular vesicles containing tight junction proteins in cells treated with either NFκB inhibitor. This suggests that one or more factors downstream of NFκB are required for assembly and/or stability of alveolar epithelial tight junctions.

497/B444
The Subcellular Localization of ZO-2 Is Regulated by the Cell Cycle and PKCε Phosphorylation.
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ZO-2 is a dual localization protein that in confluent monolayers localizes at tight junctions while in sparse cultures is also conspicuously present at the nucleus. Here we have explored if the subcellular localization of ZO-2 changes during the cell cycle and analyzed the destiny of newly synthesized ZO-2. To analyze the presence of ZO-2 at different stages of the cell cycle we arrested the cells at G0 by serum deprivation, at late G1 with mimosine and at the start of mitosis with nocodazole. Fluorescence observation of ZO-2 and Facs analysis reveal that ZO-2 enters the nucleus at a late stage of G1 and leaves the nucleus during mitosis. We also observe that in sparse cultures a significant amount of newly synthesized ZO-2 goes into the nucleus and is later relocated to the plasma membrane. Departure of ZO-2 from the nucleus is regulated by nuclear exportation signals (NES). Ser369 located within NES-1 is a putative protein kinase C (PKC) phosphorylation site. Therefore we tested the effect of PKC inhibition and stimulation on the nuclear export of ZO-2. Our results indicate that the departure of ZO-2 from the nucleus is regulated by phosphorylation at Ser369 by novel PKCε.

498/B445
Bacterial Quorum Sensing Effects on Epithelial Cell Structure and Protein Expression.
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The intestinal epithelium forms a selective permeability barrier between the lumen and the submucosa. It is maintained via complexes of cytoplasmic and transmembrane proteins composing the tight junctions (TJ) and the adherens junctions (AJ) connected to the cell cytoskeleton. The epithelial integrity can be regulated by a number of agents, including microorganisms, toxins, immune cells, cytokines, and growth factors. We have been recently shown that the Pseudomonas aeruginosa quorum sensing signaling molecule 3O-C12-HSL disrupts the barrier integrity in human epithelial Caco-2 cells and causes changes in expression and post-translational modifications of both TJ and AJ proteins. In the present study, we identified new proteins that can be important for the structure of junctions in human epithelial Caco-2 cells after treatment with P.aeruginosa 3O-C12-HSL. Using immunoprecipitation and MALDI-TOF-MS analysis, we found that the junction proteins ZO-1 and ZO-3 formed an association with alpha-actinin 4, villin, HSP90, calpain and actin in 3O-C12-HSL-treated Caco-2 cells. Another junction protein, JAM bound to Gap junction alpha-1 protein, actin, tropomyosin and myosin after administration of 3O-C12-HSL. In parallel, we showed that E-cadherin and beta-catenin were immunoprecipitated in association with myosin, actin, tropomyosin, Ca2+ binding protein,
connexin and lamin A/C. All of these proteins are important in cell proliferation and cell-cell communication, and our findings suggest that their disruption by P. aeruginosa 3O-C12-HSL quorum sensing signaling might lead to bacterial invasion and apoptosis.

499/B446
Dynamic Regulation of Septate Junction Formation during Morphogenesis.
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A key function of epithelial cells is to isolate internal and external environments by forming a tight seal between cells. This function is provided by the tight junction (TJ) in vertebrate epithelia, while in invertebrates it is provided by the septate junction (SJ). Epithelial polarity is essential for formation of both structures, but the molecular mechanisms that establish these barriers are still largely unknown. In this study we examined the dynamic behavior of four GFP-tagged SJ components (ATPa, Nrv2, Nrg and NrxIV) using fluorescence recovery after photobleaching (FRAP). Our results indicate that the SJ is an exceptionally stable and immobile structure, showing recovery times much slower than the adherens junction component E-Cadherin. In contrast Dlg, a component of the basolateral polarity complex that colocalizes with the SJ, turned over more rapidly than SJ proteins, suggesting that it is not in the same protein complex as other SJ components. Previous electron microscope analyses have shown that electron dense SJ structures initially appear at stage 14. Preceding this, SJ proteins started to be immobilized at the apico-lateral region immediately after completion of germ band retraction. To further dissect the molecular interactions within the SJ, we analyzed the stability of GFP-tagged SJ proteins in the background of mutations in other SJ genes. GFP-SJ proteins mislocalized and became more mobile in nrv2, atpα, cor, nrxIV, nrg, mega, and sinu mutants, suggesting that all of the encoded proteins form an interdependent multi-protein complex. In contrast Gli was necessary for localization of the SJ proteins but dispensable for their stability. In addition, SJ components accumulated in endocytic compartments in some mutant backgrounds, suggesting that endocytosis may play an important role in SJ formation or maintenance. Taken together these data suggest a model for SJ assembly in which a stable ‘core SJ complex’, including ATPα, Nrv2, Cor, NrxIV, Nrg, Mega and Sinu, is formed just after germ band retraction, possibly in association with membrane trafficking, and then sorted to the correct membrane domain and assembled as a highly-organized stable structure through the function of Gli.

Nuclear Architecture (500 – 511)

500/B447
Nopp140 Gene Deletion in Drosophila Melanogaster.
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Nopp140 is a nucleolar protein with multiple roles in ribosome biogenesis. Previous work in our lab (Cui and DiMario, 2007, Mol. Biol. Cell 18: 2179-2191) showed that a ≥50% reduction in Nopp140 mRNA, resulted in late larval lethality, while a 30% reduction resulted in Minute-like phenotypes that included delayed development, deformed adult wings, legs, and tergites, and occasionally missing or reduced thoracic bristles. To further characterize Nopp140 in Drosophila, we used the FLP-FRT recombination technique (Parks et al., 2004, Nat. Genet. 36: 288-292) to delete the Nopp140 gene. Genomic PCR, RT-PCR, and immuno-fluorescence microscopy all showed the loss of Nopp140 gene expression. Compared to the parental transposon-bearing embryos used for deletion recombination, Nopp140−/− embryos had similar hatching rates, but the resulting larvae died in the second instar stage after surviving for 8 days. Nucleoli were apparent by phase-contrast microscopy in Nopp140−/− cells with no apparent morphological defects, but the nucleolar rRNA methyl-transferase, fibrillarin, had a greater distribution in the nucleoplasm in Nopp140−/− cells than in control cells, suggesting that the loss of Nopp140 disrupted nucleolar function. Transmission electron microscopy showed that Nopp140−/− larvae had reduced cytoplasmic ribosomes, but contained excess nuclear virus-like copia particles, autophagosome-
like cytoplasmic structures, and many electron dense ~40 nm particles that we hope to show are RNP stress granules. Overall, our study suggests that Nopp140 is not required for morphological integrity of nucleolus, but that is necessary for proper nucleolar function, and that its deletion causes cell stress leading to larval growth arrest and eventual lethality.

501/B448
Net1-Mediated RhoA Signaling in the Nucleus Is Involved in the DNA Damage Response.
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Rho-GTPases are a family of proteins that control a wide range of different processes inside the cell, including cell survival, proliferation, gene expression, migration and death. Rho-GTPase activity is regulated by guanine nucleotide exchange factors (GEFs, which activate GTPases) and GTPase activating proteins (GAPs, which inactivate GTPases). Rho proteins reside mainly in the cytosol and are targeted to the plasma membrane (PM) upon activation. Accordingly, most GEFs and GAPs reside in the cytosol or PM of cells. However, some GEFs for the GTPase RhoA (like Net1 and Ect2) are localized to the nucleus of cells where they are assumed to exist as an inactive reservoir. Here, we show that the majority of nuclear-localized Net1 is in an active form and can induce nuclear RhoA activation upon overexpression. Furthermore, when cell are exposed to ionizing radiation (IR), which has been previously shown to activate total cellular RhoA activity, the activity of the nuclear pool of both RhoA and Net1 increases significantly, while cytosolic RhoA activity is not affected. This IR-mediated RhoA activation is inhibited by silencing Net1 expression, which causes a reduction in the amount of nuclear-localized RhoA, and a proportional decrease in nuclear RhoA activation. These results demonstrate for the first time a role RhoA and its exchange factor Net1 within the nucleus of the cell and suggest they may function in the DNA damage response.

502/B449
Fibroblast Growth Factor - 2 (FGF-2) Is a Regulator of Nuclear Body Structure.
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Nuclear bodies are distinct subnuclear structures. The survival of motoneuron (SMN) gene, which is mutated in patients with the neurodegenerative disease spinal muscular atrophy (SMA), is a marker protein for one class of nuclear bodies denoted nuclear gems. SMN has also been found in Cajal bodies, which colocalize with nuclear gems in many cell types. Interestingly, SMA patients display a reduced number of nuclear gems. Little is known about the regulation of nuclear body formation and stabilization. We have previously shown that a nuclear isoform of the fibroblast growth factor FGF-2 [FGF-2(23)] binds directly to SMN. In this study, we analyzed the consequences of FGF-2(23) binding to SMN with regard to nuclear body formation. on a molecular level, we could show that FGF-2(23) competed with Gemin2 - a component of the SMN complex, which is necessary for nuclear gem stabilization - for binding to SMN. on a cellular level, FGF-2(23) decreased the number of SMN-positive nuclear bodies. Knock-down of Gemin2 by siRNA caused destabilization of SMN-positive nuclear bodies. The same effect could be observed In Vivo in motoneurons of FGF-2 transgenic mice compared to their wild-type littermates. In addition, we analyzed the nuclear FGF-2(23) complex. The competition of FGF-2(23) and Gemin2 for binding to SMN is reflected on a cellular and In Vivo system level by a negative regulative function of FGF-2 in nuclear body formation. This study is the first demonstration of a new functional role of a growth factor in regulating structural entities of the nucleus. References: Claus P., Döring F, Gringel S, Müller-Ostermeyer F, Fuhlrott J., Kraft T., Grothe C. (2003) Differential Intranuclear Localization of Fibroblast Growth Factor - 2 (FGF-2) Isoforms and Specific Interaction with the Survival of Motoneuron Protein. J. Biol. Chem., 278:479-485. Bruns, A.F., van Bergeijk J., Lorbeer C., Nölle A., Jungnickel J., Grothe C., Claus P. (2009): The Growth Factor FGF-2 Regulates the Stability of Nuclear Bodies. Proc. Natl. Acad.
**503/B450**

Treatment with Farnesyltransferase Inhibitors Causes Donut-Shaped Nuclei Due to a Defect in Centrosome Separation.

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Farnesyltransferase inhibitors (FTIs) were initially developed as anticancer drugs targeting Ras oncogenes and are now also used to treat patients with Progeria. Anecdotal observations suggest that FTIs cause donut-shaped nuclei. Objective: to identify the mechanisms underlying “donut-formation” and the effects of this nuclear abnormality on cell function. Methods: Donut-formation was monitored by time-lapse microscopy of primary human skin fibroblasts or HeLa cells expressing GFP-tagged lamin a and HEp2 laryngeal cancer cells co-expressing GFP-tagged histone-3 and mcherry-β-tubulin. Photo-conversion of Dendra-tagged histone-4 was used to evaluate the effect of donut-nuclei on proliferation. Results: FTI-treatment caused donut-shaped nuclei in 5-30% of skin fibroblasts, HeLa and HEp2 cells. Importantly, donut-nuclei were found in gut, skin, and blood vessel tissue sections of FTI-treated mice. Time-lapse imaging revealed that donut-shaped nuclei arose upon mitosis; blocking mitosis eliminated donut-formation. Strikingly, 70% of donut-nuclei were found in binucleated cells. Further analysis suggested a defect in centrosome separation as the primary cause. at interphase, 95% of these binucleated cells had a single centrosome-cluster located in between both nuclei. In mid-anaphase, however, multiple centrosomes could be seen close to or in the evolving donut-hole. A thick bundle of microtubules interconnected the holes of developing donut-shaped nuclei. In 35% of FTI-treated cells, centrosomes were located in between both daughter nuclei, i.e. facing the equatorial plane, in late anaphase. Overall, the distance between the centrosomes was smaller in FTI-treated cells, both at metaphase and anaphase. Although the donut-shape did not affect the rate of division, 60% of them divided abnormally; 95% of binucleated fibroblasts did not divide over a 4-day period. Furthermore, FTI-treatment increased the number of aneuploidic cells. Conclusions: FTI-treatment interferes with centrosome separation at mitosis and thereby, causes donut-nuclei and binucleation. The resulting proliferation defect could contribute to the anticancer effect of FTI, though warrants caution for its use in the treatment of Progeria.

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**504/B451**

Lamin Assembly Is Governed by the Interaction of Multiple Charged Clusters: Formation of Extended Networks in Two Dimensions.

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Lamins are intermediate filament (IF) proteins specialized to form two-dimensional networks at the inner nuclear membrane (INM) as impressively represented in the giant cell nuclei of Xenopus oocytes. The establishment of such regular and extended lamin fiber systems as well as their degree of connectivity may be extensively regulated by factors within the INM, such as emerin and lamin B receptor (LBR), along with interactions that occur specifically in somatic cells, i.e. association with components of interphase chromatin such as histones and the barrier-to-autointegration factor (BAF). Recently, mutations in lamin a have been demonstrated to cause a bewildering number of different human diseases that may in part result from structural changes of the nuclear envelope but may also affect the specific association with distinct signaling molecules and transcription factors. In addition, it has been hypothesized that a widespread nucleoplasmic
A-type lamin system may functionally organize nuclear architecture. We have now established conditions to assemble recombinant lamin a filaments in a dilution-type fashion similar to the one successfully previously utilized for cytoplasmic IF proteins. Thereby we are able to analyze the assembly properties of wildtype versus mutated lamin A. Hence, by diluting the soluble, dimeric lamin complexes into buffers of appropriate composition, we are able to pilot their assembly process into different structural pathways. In addition, by restricting the spatial parameters to a near two-dimensional situation, we are able to enhance different assembly modes in a directed fashion. Moreover, we can influence the networking interactions of fiber-type structures by the addition of reactive fragments of several INM proteins. Here we worked out conditions that sharply distinguish wildtype lamin a from the progeroid mutant lamin E145K.

505/B452
Dynamics of Telomere Clustering in Yeast.
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For yet unclear reasons, telomeres form small but dynamical clusters. We study here the clustering of 32 independent telomeres modeled as Brownian object moving inside the restricted cellular domain. When the average number of telomere clusters is 5, we find that the dissociation constant of a telomere packet, regardless of its size in 3D is around 20s. By comparing several different models, we suggest that in a cluster, telomeres should all be attached to a unique docking zone that we characterize. Finally, we study the dynamics of two independent telomeres and show that at equilibrium, when the mean number of clusters is constant: two individual telomeres are highly dynamic and we obtain the intermittent law of switching between clusters. We conclude that telomere clustering is a highly dynamical process in Yeast Cerevisiae and we suggest similar results for Plasmodium falciparum telomeres.

506/B453
Accurate, Efficient and Reproducible Electrophoretic Determination of the Mammalian Nuclear Matrix Proteome and Nucleic Acid Composition Using a Phastsystem.
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Nucleic acid metabolism is biochemically compartmentalized to the nucleus and it is necessary to define the proteome of the different macromolecular structures within this organelle. for that reason, a reproducible and efficient characterization of specific sub-nuclear structures is required. We have isolated the nuclear matrix (NM) fraction from rat liver nuclei by sequential centrifugation steps at 13,000 X rpm, staggered between an endogenous nuclease enzymatic treatment for 2 hours at 37 °C, followed by high salt (2.0 M NaCl) and non-ionic detergent extractions (0.1% Triton X-100) to eliminate the bulk of chromosomal DNA/RNA, chromatin histone proteins and the double lipid bilayer of the nuclear envelope, respectively. The integrity of intact rat liver nuclei, the NM, and the nuclear envelope structures was confirmed by electron microscopy. Upon demonstration of the high level of purity of these nuclear structures, we proceeded to run the NM extract on a 20% polyacrylamide gel, under SDS-denaturing electrophoretic conditions, using the phastsystem in less than 2 hours. We reproducibly observed the absence of histone proteins and the abundant presence of the characteristic NM protein lamins by Coomassie Blue staining in the region of 60-70 kD. By contrast, when we stained an identical gel with silver, following electrophoretic separation using a Tris-Borate-EDTA buffer, we observed the presence of significant amounts of NM associated nucleic acids, e.g., DNA, RNA, and more importantly protein-free ADP-ribose polymers. While ADP-ribose polymers are found in significantly lower concentrations than DNA and RNA (3-4 orders of magnitude lower concentration) in the NM, ADP-ribose chains could be purified by affinity chromatography on a boronate resin prior to electrophoresis on the phastsystem (Alvarez-Gonzalez, et al., 1983). As a result, we significantly improved the electrophoretic resolution to a highly efficient and reproducible detection of the
ADP-ribose polymer content associated with the NM fraction, after chemical release from NM proteins, under the mild alkaline pH conditions required for chromatographic purification.

507/B454
The Role of Nup358 in Nuclear Import of the Tumor Suppressor DBC-1.
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Nucleocytoplasmic transport occurs through nuclear pore complexes (NPC) and is mediated by soluble transport receptors. Some nucleoporins, components of the NPC, have been suggested to function as initial or terminal binding sites for transport complexes. The 358 kDa nucleoporin Nup358/RanBP2 is a major component of the cytoplasmic filaments of the NPC. Earlier findings had suggested that Nup358 is dispensable for nuclear protein import. We could demonstrate, however, that depletion of Nup358 by RNA-interference leads to a reduced import rate for the importin α/β- as well as the transportin-dependent protein transport. Among others, we identified the tumor supressor DBC-1 (Deleted in Breast Cancer 1) as a protein, whose nuclear localization depends on Nup358. It is transported into the nucleus in an importin α/β-dependent manner. A putative coiled-coil region in DBC-1 appears to be required for its Nup358-dependent import. Furthermore, different regions within Nup358, which might be involved in DBC-1-import, are analyzed. Full-length Nup358 as well as C-terminal deletion mutants, expressed from RNAi-insensitive mRNAs, are able to rescue the observed import defects in cells depleted of endogenous Nup358. Together, our results point to an important role of Nup358 as a general docking site for specific incoming transport complexes.

508/B455
A Relationship between Yeast Nuclear Morphology, Large Structural Nucleoporins, and the Sun Domain Protein MPS3.
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Nuclear architecture regulates essential functions of the nucleus. Yet little is known about the genes that control nuclear structure or the mechanisms by which they affect nuclear function. In order to explore the relationship between nuclear structure and function in budding yeast, we are using both high-throughput and candidate approaches to study mutants with abnormal nuclear morphology. Cells carrying a deletion of the non-essential SPO7 gene misregulate phospholipid biosynthesis and exhibit expansion of the endoplasmic reticulum and nuclear envelope, causing aberrant nuclear morphology. In order to identify aspects of nuclear function that are compromised in this mutant, we have used a combination of approaches to identify genes whose disruption confers synthetic lethality with the spo7Δ allele. Temperature-sensitive alleles of the essential SUN domain gene MPS3 exhibited synthetic lethality and changes in nuclear morphology in combination with the spo7Δ allele. The Mps3 protein resides at the spindle pole body (SPB) and around the nuclear periphery, and MPS3 has been implicated in SPB duplication as well as various aspects of nuclear architecture. Using a high-copy suppressor screen, we have identified genes that rescue the synthetic lethality between spo7Δ and mps3 alleles, including two genes encoding large structural nucleoporins. Our data suggest that these nucleoporins act in a dominant negative manner to rescue a defect in MPS3 function. Ongoing studies aim to determine the biological basis of the synthetic lethality between SPO7 and MPS3, and to elucidate the relationship between these large structural nucleoporins and MPS3 function.

509/B456
DNA Zip Codes: An Ancient Mechanism for Gene Targeting to the Nuclear Periphery.
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Many highly expressed genes are dynamically recruited to the nuclear periphery upon transcriptional activation in Saccharomyces cerevisiae. We have identified two Gene Recruitment Sequences (GRSs) from the promoter of the INO1 gene that target the gene to the nuclear periphery upon activation. The GRSs function as DNA zip codes; they are sufficient to target a nucleoplasmic locus to the nuclear periphery through interaction with the nuclear pore complex. Full transcriptional activation of INO1 requires GRS-mediated targeting of the promoter to the nuclear periphery. Finally, a GRS functions as a DNA zip code in Schizosaccharomyces pombe as well, suggesting that this mechanism of targeting to the nuclear periphery is conserved over at least one billion years of evolution.

510/B457

Phosphorylation of Nup1 by Cdk Regulates the Localization of Active Genes to the Nuclear Periphery through the Cell Cycle.

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Upon activation, a number of inducible genes in yeast are targeted to the nuclear periphery and physically interact with the nuclear pore complex. Using a quantitative chromatin localization assay, we find that the peripheral localization of the active INO1 and GAL1 genes is regulated through the cell cycle. Both genes localized at the nuclear periphery during G1, became nucleoplasmic during S-phase and returned to the nuclear periphery during G2/M. Relocalization of these genes to the nucleoplasm during S-phase required the initiation of DNA replication. The loss of peripheral localization required the cyclin dependent kinase inhibitor (CKI) Sic1 and could be induced during G1 by activation of the CKI Far1. These observations suggested that phosphorylation of a substrate by cyclin-dependent kinase (Cdk) promotes peripheral localization. Indeed, we found that two Cdk phosphorylation sites in Nup1 and the Cdc28 kinase were required for peripheral targeting of active INO1 and GAL1. Furthermore, introduction of aspartic acid residues in place of either of these two sites in Nup1 bypassed the requirement for Cdc28 and resulted in targeting of INO1 and GAL1 to the nuclear periphery during S-phase. Thus, cyclic phosphorylation of a nuclear pore component by cyclin dependent kinase coordinates gene localization and transcriptional activity with DNA replication.

511/B458

Cysteine Reactivity as a Probe of the Nuclear Proteome: An In Situ Measurement of the Tolerance of Lamin A/C to Stress.


A range of diseases and syndromes can be traced to mutations to the gene *lmna*, which codes for the nuclear intermediate filament proteins, Lamin A and Lamin C. Emery Dreifuss Muscular Dystrophy is the consequence of a single amino acid substitution (R453W) to the c-terminal immunoglobulin-like (Ig) domain of the Lamin A/C proteins. Solution studies of the isolated domain have suggested that the disease mechanism could result from partial unfolding of the Ig domain at physiological temperatures. In order to make corresponding measurements of thermal stability within structurally intact nuclei, we introduce a novel assay in which the fold-state of a protein is probed by measuring the extent and kinetics of cysteine labeling. The lone cysteine within the Ig domain reacts slowly in a covalent reaction with the fluorescent dye bromobimane (mBBr) under native conditions, but this reaction is accelerated beyond Arrhenius behaviour as the protein unfolds and the cysteine residue is exposed at elevated temperature. By using LC/MS/MS to quantify the extent of labelling, we are able to extend this methodology to profile the response to thermal stress of a large number of nuclear proteins in parallel.

Ribonucleoproteins (512 – 526)

512/B459
Poly(ADP-ribose) Is Required for Stress Granule Integrity and Regulates microRNA Activity.
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Cells respond to external stress via reprogramming of gene expression. One way to cope with stress, such as heat shock, oxidative stress, ischemia or viral infection, is to assemble large multiprotein complexes called Stress Granules (SGs). SGs contain stalled translation initiation complexes, poly(A)+ mRNAs, and their associated proteins, such as G3BP1, TIA1, and the microRNA(miRNA)-binding protein Ago2 which I previously identified as a component. SGs are thought to regulate the stability and translation potential of mRNAs, but the mechanism of SG function and the regulation of SG assembly are largely unknown. Here I will present that Poly(ADP-ribose) (pADPr) - a large, branched, negatively charged polymer - localizes to SG and is required for SG integrity. Moreover, I identify 5 specific pADPr polymerases (PARPs) and 2 pADPr glycohydrolase (PARG) isoforms as SG components - the first identification of multiple enzymes regulating pADPr function in one specific cellular activity. I will discuss how Poly(ADP-ribose) (pADPr) acts as a scaffold of SGs and its implication in assembly and disassembly of this organelle. All known de novo nucleators of SG assembly, including Ago2, G3BP1 and TIA1, are directly modified by pADPr and such modification increases upon stress. I will also present data on how this modification modulates one form of the post-transcriptional gene regulation - miRNA activity - in normal and stressed conditions. These results further expand the role of pADPr in responding to DNA damage in the nucleus and extend its function to the cytoplasm in modulating post-transcriptional gene regulation. The understanding of the cytoplasmic role will likely impact the current treatment in cancer, ischemia and inflammation diseases using PARP inhibitors, which are actively developed by pharmaceutical companies.

513/B460
Map Kinase Signaling Regulates mRNA Export during Cellular Stress.
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Gene expression in eukaryotes requires transcription, messenger RNA (mRNA) processing and nuclear mRNA export prior to translation. Transport of mature mRNAs occurs through nuclear pore complexes embedded in the nuclear envelope. It is well established that heat shock and other cellular stresses result in alteration of mRNA export, however the precise mechanism for transcript-specific regulation of transport is unknown. Using the budding yeast S. cerevisiae as a model system, we analyzed a panel of mRNA export factors for changes in cellular localization upon heat shock. Strikingly, we found that the mRNA-binding protein Nab2 forms nuclear foci upon heat shock. Foci formation was dependent on the NPC-associated protein gene MLP1. We also found that Nab2 was phosphorylated after shifting cells to heat shock. Interestingly, phosphorylation was independent of nuclear foci formation, suggesting that these processes reflect distinct cellular roles. By screening through the S. cerevisiae null collection, we identified a MAP kinase pathway responsible for the phosphorylation. In Vitro kinase assays showed that the MAP kinase directly phosphorylated recombinant Nab2. To determine the Nab2 phosphorylation sites, Nab2-TAP was isolated from heat shocked cells and mass spectrometry was conducted. Two specific sites were defined and confirmed by mutagenesis. Using yeast genetics, we are currently analyzing the requirements for phosphorylation in Nab2 foci formation and differential mRNA export. Overall, because Nab2 functions in both polyadenylation and mRNA export, we speculate that alteration of Nab2 promotes retention of non-heat shock transcripts potentially by altering mRNA 3’ end processing and/or export. This represents a novel mechanism for regulating mRNA binding proteins during differential mRNA export. Supported by NIH R01 GM051219 to S. R. W.

514/B461
RNA Interference and Plant-Fungal Symbiosis- Characterization of Argonaute Genes from Laccaria bicolor.
RNA interference (RNAi) is a conserved group of regulatory mechanisms in which small regulatory RNA molecules serve as guides for protein complexes to suppress expression of targeted gene sequences. Argonaute is one of the key proteins in RNAi and is the catalytic cleaving component of the RNA induced silencing complex. Argonaute and other RNAi components have been found to have a role in fungal gene silencing, known as quelling, and is similar to RNAi in animals. The biogenesis of small regulatory RNAs involved in RNAi in fungi have yet to be identified. Using ectomycorrhizal symbiotic fungus *Laccaria bicolor* (*L. bicolor*) as a model, my objective is to identify the genes for biogenesis of small regulatory RNAs and determine their role in gene regulation. I have cloned and sequenced full length cDNAs of all three hypothetical Argonaute genes from *L. bicolor* during early interaction phase of symbiosis. via quantitative PCR analysis, I have also confirmed their interaction-specific high expression which suggests a possible regulatory role for Argonautes in symbiosis. Biochemical characterization of recombinant *L. bicolor* Argonaute proteins was determined through In Vitro RNA cleavage assays. Complementation of a *Schizosaccharomyces pombe* Argonaute mutant was attempted to establish the functional role of *L. bicolor* Argonaute genes. Our results strongly suggest RNAi machinery are likely involved in the production of small regulatory RNAs involved in symbiosis formation. This data will enable us in the future to elucidate the critical biological role played by RNAi machinery in *L. bicolor* and also its role in formation of symbiosis.

515/B462

**Non-Coding ncRNA-Protein Interaction Networks, a “Rosetta Stone” to Decipher the Epigenetic Code and Algorithm.**

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OBJECTIVE: From the same genotype, the genome orchestrates genetic [Mendelian] and epigenetic [non-Mendelian] information into different organized or mess-chaotic [tumor] phenotype variations. By genetic complimentary and triplet codes, transcription and translation of DNA genes into proteins matter only ~2% of transcriptional output. Functions of residual ~98% transcriptional RNA output of cells activated by extrinsic [environmental] factors were investigated on structural codes for episcription [greek: “overscription”]. METHODS: Ann.N.Y.Acad.Sci.1022:163-184,2004; 1137:316-342,2008. RESULTS: Functional edited, modified, redox- and metalloregulated small hairpin ncRNA [<200n] as bioaptamers in RNP complexes were isolated from epigenetic remodeling variation models directed to proteinaceous gene expression and regulation rather than genomic DNA sequence changes. Some are not complimentary to protein-coding DNA sequences. They may address defined homologous helix-nucleating domains shared in epigenetic regulator proteins entangled in tolerated growth, vascularization, metabolic syndromes, cancer and epigenetic and genetic information indexing of the epigenome. at variance to genetic codes, ncRNA are not “non-coding”, but have to be read in another “language alphabeth”. They code algorithmic [necessity] rather than stochastic [chance] and heuristic [trial/error] regulatory processes. CONCLUSIONS: The results suggest the epigenetic code consists of different intrinsic and extrinsic interactive imprints and factors: [1] Non-Mendelian nucleic acid 3D-episcripts. Some are non-complimentary ncRNA derivatives of genomic DNA. [2] Conserved homologous domains in epigenetic regulator protein and nucleic acid matrices of Mendelian genotype origin in helix-nucleating interaction with [1], comprising variant and heritable disease implications. [3] Extrinsic and intrinsic factors upon which formation of [1] and interaction with [2] depends [e.g. redox- and metalloregulation]. Thus, the epigenetic code comprises more diversity, complexity and plasticity repertoires than genetic codes. It conforms to Darwin-Mendel’s principles in synergism with some [Lamarck’s] environmental influences for epigenetic imprinting and inheritance.

516/B463
O-GlcNAc Cycling Enzymes Associate with the Translational Machinery and Modify Many Core Ribosomal Proteins.
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Post-translational modifications of translation factors represent essential mechanisms for regulating global protein synthesis. High-throughput studies have identified several translation factors and ribosomal proteins (RPs) that are modified by O-GlcNAc. Employing subcellular fractionation, followed by galactosyltransferase labeling with UDP-[3H]Gal or Western blotting, we find that O-GlcNAc is present on many proteins from actively translating polysomes. Using reverse phase-high pressure liquid chromatography (RP-HPLC) and MS/MS we identify at least twenty GlcNAcylated RPs, of which only seven had been previously reported. Chemoenzymatic labeling combined with β-elimination followed by Michael addition with DTT (BEMAD), allow us to map sites of O-GlcNAc modification on four RPs (RPL6, RPL29, RPL32 and RPL36). RPS6, a component of the mammalian target of rapamycin (mTOR) signaling pathway, follows different dynamics of GlcNAcylation than nutrient-induced phosphorylation. In addition to GlcNAcylated RPs translational preparations also contain both O-GlcNAc cycling enzymes. O-GlcNAc Transferase (OGT) and O-GlcNAcase (OGAse) strongly associate with ribosomes. Both enzymes are present in sub-polysomal fractions (40S, 60S and 80S monosomes), as well as in light and heavy polysomes. Immunofluorescence microscopy shows that OGAse, but not OGT, is present within the nucleolus and co-localizes with fibrillarin, a ribosome-processing factor. Disruption of nucleolar structure by inhibition of rRNA synthesis results in loss of OGAse staining within the nucleolus. Adenoviral-mediated over-expression of OGT, but not of OGAse or GFP control, causes a dramatic accumulation of 60S subunits and 80S monosomes. Our results not only establish that GlcNAcylation extensively modifies RPs, but also suggest that O-GlcNAc may play important roles in regulating translation and ribosome biogenesis. Supported by NIH grants R01 DK61671 and R01 CA42486. Dr. Hart receives a share of royalty received by the university on sales of the CTD110.6 antibody. Terms of this arrangement are managed by JHU.

517/B464
microRNA Activity Is Regulated by Poly(ADP-ribose).
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microRNAs (miRNAs) are a class of ~22 nucleotide non-coding RNAs predicted to regulate at least 50% of mammalian mRNAs. Emerging data suggest that some miRNAs are instrumental for cellular stress responses by regulating the stability and translation of existing mRNAs post-transcriptionally through the activity of Argonaute protein. I previously demonstrated that Argonaute/miRNA complexes localize to cytoplasmic organelles called Stress Granules (SGs), which assemble immediately upon stress stimuli such as heat shock, oxidative stress, hypoxia, UV irradiation or hypoglycemia. SGs contain stalled translation initiation complexes, poly(A)+ mRNA and their associated RNA-binding proteins (RBPs). During normal, non-stress conditions, miRNA targets are typically repressed and translation occurs preferentially on non-miRNA targets. Here I will present data showing that a member of the Argonaute family, Ago2, is directly modified by poly(ADP-ribose) (pADPr) - a large, branched, negatively charged polymer - during both normal and stressed conditions. As a result of the modification, the proportion of miRNA targets being translated relative to non-miRNA targets increases when translation becomes limiting upon stress. I also show that the modification is mediated through the activity of 2 SG-localized, pADPr polymerases (PARPs) that co-bind with Ago2 to miRNA targets. These results identify a new mechanism for attenuating silencing by miRNA during stress. In addition to Ago2, I also identified other SG-localized RBPs as targets of increased pADPr activity upon stress. I will conclude by discussing how pADPr could reprogram the expression of pre-existing mRNAs selectively and immediately as a general mechanism for cells to respond stress.

518/B465
The Paraneoplastic Neurologic Disease Antigen Nova Regulates Transcript Levels through Occult Nmd Exons and 3'UTR Interactions.

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RNA binding proteins (RBP) play many important roles to regulate the alternative splicing, translation, stability and localization of RNAs. It has been known that Nova plays a role as a splicing factor in neurons through previous work. Nova proteins do shuttle between nucleus and cytoplasm by using a Nuclear Localization Signal and a Nuclear Export Signal and it suggests to have other functions in the cytoplasm besides the splicing in nucleus. Immunohistochemical and biochemical studies have demonstrated that Nova proteins are enriched in the nucleus and also distributed in the cytoplasm. A new method, HITS-CLIP (high-throughput sequencing crosslinking immunoprecipitation) has identified many In Vivo RNA targets through covalent cross-linking of Nova proteins and RNAs. Sub-fractionationed HITS-CLIP can show live Nova-RNA interaction sites in nucleus and cytoplasm, respectively. Microarray data showed transcripts levels of many Nova’s target RNAs were not only up-regulated but also down-regulated in KO. Distribution of HITS-CLIP tags among top 229 transcripts exhibited 3'UTR in the minority of instances (23.9%), and instead the dominant location of CLIP tags was intronic. It suggests the possibility that Nova act through occult elements with annotated introns. RT-PCR and sequence analysis showed there are many unknown Nova-dependent alternative splicing events which can remove or introduce premature stop codons. It can turn off/on the nonsense-mediated decay (NMD) pathway regulating gene expression posttranscriptionally. Nova proteins are important to set a balance of many protein expression through this mechanism. To determine whether these genes regulated by Nova proteins is NMD-dependent, siRNA to upf1 and emetine drug were used to block NMD pathway and then see if transcript levels were rescued. Additionally, cytoplasmic fractionationed HITS-CLIP showed many tags in 3'UTR and these YCAY elements among transcripts having mild change only in DKO are involved to increase mRNA stability. It is the first example to demonstrate that an RBP, Nova, up/down-regulates transcripts through occult NMD exons and up-regulates transcripts through 3'UTR interactions as well.

Exploring Template Utilization by Telomerase with Saccharomyces cerevisiae Yeast.

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<T> Telomerase is a reverse transcriptase enzyme required for the maintenance of simple DNA repeats at the ends of most linear eukaryotic chromosomes (telomeres). The catalytic core of yeast telomerase includes the Est2 protein and the TLC1 RNA. We have shown that mutations in the N-terminus of Est2p cause telomere over-elongation and alter the sequence of the telomere repeats (Ji et al., 2008). This change in telomere sequence is seen in strains expressing est2 alleles that cause telomere over-elongation through genetically distinct pathways (e.g. est2-LTE76K is RAP1 dependent; est2-up34 is PIF1 dependent) suggesting that an altered telomere sequence may be a consequence, rather than a cause, of over-elongation. RAP1 Interacting Factors 1 and 2 (RIF1 and RIF2). Our data show that the telomere sequences of these strains are similar to those of WT. The altered sequence phenotype is also not seen with mutations in genes that indirectly regulate telomere length, ELG1 and SSN8. These data suggest that the altered telomere sequence and length phenotypes are separate. est2 mutations that affect telomere length through different pathways give rise to similar changes in telomere sequence may be reconciled through understanding the mechanism through which the telomere sequence is generated. We know that the sequence change generated by est2-LTE76K is not due to an increased processivity of nucleotide addition by mutant telomerase (Ji et al., 2008). Rather, the est2 alleles may change alignment of the TLC1 RNA with the DNA substrate. To test this hypothesis, I have incorporated tlc1 template mutants in the WT and est2 strains. Our data show that the sequences added by WT and mutant telomerase are drastically affected by changes in the template region, supporting our hypothesis. We are in the process of examining telomere addition to a short telomere seed In
Vivo and monitoring activity of WT and mutant telomerase on a series of differing DNA primers in vitro.

520/B467
Novel Role for Nup98 as a Component of a Regulatory Germline RNP.
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Germ granules are cytoplasmic ribonucleoprotein complexes essential for differentiation and function of germ cells. Germ granules are often found at the cytoplasmic face of the nuclear envelope, close to nuclear pores (Strome and Wood 1982; Pitt et al., 2000; Pavrinen et al., 2005). To investigate a potential link between nuclear pore components and germ granules, we performed an RNAi screen depleting annotated nucleoporins in C. elegans. We found that Nup98/96, Nup153, and Nup155 are essential for germ granule integrity and perinuclear localization. Localization analysis suggested that Nup98 is unique among these proteins as it localizes to the germ granules in addition to its expected localization at the nuclear pores. Importantly, Nup98 localization to germ granules is disrupted when Nup155 or Nup153 are depleted. We have found that Nup98 critically contributes to germ granule function. *nos-2* codes for a translationally-repressed mRNA that localizes to germ granules. In the absence of Nup98, *nos-2* mRNA no longer associates with P granules and is translated precociously. Immunoprecipitation of Nup98 brings down *nos-2* mRNA, suggesting that the two exist in a repressive complex in germ granules. Consistent with this hypothesis, tethering of Nup98 to the 3’ UTR of a heterologous mRNA causes its translational repression in Hela cells. Immunoprecipitation of Nup98 from mouse testes brings down MVH, a mouse germ granule protein, suggesting a conserved role in cytoplasmic mRNPs. We hypothesize that Nup98 is recruited to a subset of regulated mRNAs to direct them to repressive complexes in the cytoplasm. Nup98 has been implicated in regulation of mRNA export from the nucleus (Pritchard et al., 1999, Enninga et al., 2002). Our results support expanding Nup98’s role to include regulation of mRNA fate in the cytoplasm.

521/B468
Recognition of Ribosomal RNA by Rprotein S4.
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Ribosome assembly is a vital cellular process that demands a large amount of energy. A cell can create more than 2000 ribosomes per minute under ideal growth conditions. In yeast, rRNA transcription accounts for over 60% of all transcription, while the transcription of associated r-proteins accounts for 50% of all pol II transcription events. Ribosomal proteins are directly involved in the regulation of ribosome biosynthesis in both yeast and bacteria. In E. coli, free r-protein S4 inhibits the translation of an operon encoding S4, along with ribosomal proteins S13, S11, and L17. In addition, S4 inhibits the termination of pre-rRNA transcription. Bacterial 30S ribosome subunit assembly requires the proper folding of the 16S rRNA and the hierarchical association of over 20 individual r-proteins. S4 binds to the 5’domain of the 16S rRNA at a five-way helical junction (5WJ). Once bound to the rRNA, S4 nucleates assembly of the 30S subunit by bringing many helices in the 5’ domain into close proximity. In addition, both S4 and the 5’domain undergo changes in structure upon binding. Interactions between S4 and the 16S rRNA were probed through protein engineering and footprinting of the RNA. Recently, time resolved hydroxyl radical footprinting data from our lab demonstrated that, although early steps in ribosome assembly are linked to intrinsically stable RNA structure, later regions correspond to regions of induced fit between the protein and rRNA. A series of S4 truncation and point mutants were created at residues likely to be critical for rRNA recognition by S4. An N-terminal truncation mutant, as well as a single point mutant completely abolished 5WJ rRNA binding, while other point mutants showed varied decreases in affinity, from a 5-fold to nearly 50-fold reduction in affinity. Hydroxyl radical and SHAPE experiments are currently underway to determine the effects of the various mutant proteins on folding of the 5WJ RNA and 30S assembly.
SUNDAY

522/B469
Systematic Identification of Protein Phosphatases Regulating Localization and Function of the SMN Complex.
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Removal of introns from pre-mRNAs (splicing) requires the coordinated action of Uridine-rich small ribonucleoprotein particles (UsnRNPs). UsnRNPs comprise a RNA moiety, transcribed in the nucleus, as well as different proteins. E.g. most UsnRNPs share seven Sm proteins, which assemble around the RNA in a heptameric ring. Sm ring formation occurs in the cytoplasm and involves the Survival of Motor Neurons (SMN) Complex. The SMN complex is believed to accompany the maturing UsnRNPs back into the nucleus and to Cajal bodies, where SMN catalyzes further maturations steps in UsnRNPs. UsnRNPs then reach sites of active splicing, while the nuclear SMN complex accumulates in Cajal bodies. The specific functions of the SMN complex in the nucleus or the cytoplasm, and exchange and distribution of the SMN complex between the two compartments are expected to be tightly regulated. It is now well established that the phosphorylation state of subunits of the SMN complex differs significantly between the nucleus and the cytoplasm. We recently could show that the PPM1G phosphatase is required to maintain SMN in a hypophosphorylated form in the nucleus to allow accumulation of SMN in Cajal bodies (Petri et al., JCB, 2007). In order to systematically identify regulators of SMN localization and function, we now screened a siRNA library for the knock-down of all human phosphatase gene products. In a semi-automated screen, we determined the specific localization pattern of SMN in the nucleus and in the cytoplasm after knock-down of ca. 300 human phosphatases and phosphatase-related gene products. Besides PPM1G itself, our screen reveals several novel potential regulators of the SMN complex, which are required for proper Cajal body accumulation, distribution of the SMN complex between the nucleus and the cytoplasm and its overall stability. Our data suggest direct regulators of the SMN complex as well as integration of SMN complex function into the overall proliferation state of human cells.

523/B470
Human mRNA Decapping Enzyme, hDcp2, Stability May Be Regulated According to mRNA Substrate Levels.
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mRNA decay is a key step in the regulation of gene expression. A critical step in several mRNA decay pathways is decapping. Here we present evidence that mRNA decapping in human cells may be regulated through the stability of the decapping enzyme, hDcp2. We have observed that exogenously expressed hDcp2 is highly unstable. By contrast, all tested non-catalytic decapping factors are stable. The following lines of evidence suggest that the C-terminus of hDcp2 is critical for hDcp2 instability. First, its deletion renders exogenous hDcp2 stable and second, overexpression of Hedls/Ge-1/Edc4, a decapping factor that interacts with the hDcp2 C-terminus causes stabilization of full-length hDcp2. Interestingly, hDcp2 is stabilized by a single point mutation that renders hDcp2 catalytically inactive. This correlates with the entrapment of the decapping complex on mRNA substrates upon expression of catalytically inactive hDcp2, as evidenced by the accumulation of the decapping complex in cytoplasmic mRNP granules called P-bodies under these conditions. Based on these observations, we hypothesize that hDcp2 stability is regulated according to the levels of its mRNA substrate. This may provide a mechanism to prevent promiscuous mRNA decay by an excess of hDcp2, and to rapidly upregulate hDcp2 when conditions require decapping of a large number of mRNAs. These results provide novel insights into the regulation of mRNA decay enzymes.

524/B441
Ultrastructural Analysis of the Biogenesis of Lacandonia Granules.
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Lacandonia granules are extranucleolar ribonucleoprotein (RNPs) particles, 32 nanometers in diameter intermixed with fibrils that were first described in the plant Lacandonia schismatica but are also present in the close relative Triuris brevistytilis and the gymnosperm Ginkgo biloba. Ultrastructural cytochemical, immunocytochemical and in situ hybridization studies suggest that these particles are equivalent to perichromatin and Balbiani ring granules described in mammals and insects. However, no studies have been done to know the biogenesis of Lacandonia granules. In the present work we analyze the spatial relations between the granules and surrounding fibres in the nuclei of Lacandonia schismatica by an ultrastructural approach that includes the use of a goniometer adapted to the transmission electron microscope, to produce three-dimensional images as stereopairs. Our results show that Lacandonia granules are associated to fibrils suggesting several steps in the formation. The first one consists of very small granules associated to thick fibrils forming an amorphous cumuli most of the cases associated to chromatin. The second step consist in more abundant granules of heterogeneous size distributed along the interchromatin and perichromatin space that are intermixed with more thin fibrils and the last step consists of the 32 nm particle which is associated to thin fibers and could represent mature granules. Our results suggest that during the biogenesis of the granules, they originate from surrounding fibrils (supported by UNAM DGAPA-PAPIIT IN-206307).

525/B472
Exploring Assembly Intermediates of the 30S Ribosomal Subunit Using Single Particle EM.
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Ribosomes are self-assembling macromolecular machines responsible for translating the cell’s genetic code into the workhorses of life, proteins. Given their fundamental importance, an understanding of ribosome synthesis, regulation, and assembly is imperative. Extensive studies on the accepted model for ribosome assembly, the E. coli 30S subunit, suggest that ribosome assembly is (1) cooperative, (2) hierarchical, and (3) occurs via multiple, parallel pathways rather than through a single rate-limiting step. We have taken EM snapshots of assembling 30S subunits at 8 different time points and determined the compositions and 3D structures of more than 10 assembly intermediates. The data confirm the existence of multiple assembly intermediates that are variously populated over time and illustrates parallel assembly in the 3’ domain. This combined with previous studies suggest a mechanism for 30S assembly that includes binding dependencies and kinetics.

526/B473
3D Structure of Human Dicer by Single Particle Electron Microscopy.
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Dicer is a specialized ribonuclease that initiates RNA interference (RNAi) by cleaving double-stranded RNA into RNA fragments about 22 nucleotides in length. Dicer also serves as a scaffold for the initiation of RNAi by bringing together multiple factors required for the correct processing of small RNAs. Here, we present the 3-D structure of human Dicer bound to the protein TRBP at approximately 20 angstrom resolution determined by electron microscopy (EM). A robust and reliable 3-D model of the complex was generated using a combination of random conical tilt and projection matching refinement methods. Our analysis reveals that the Dicer-TRBP complex is an L-shaped molecule. Docking the crystal structure of Giardia Dicer, which represents the nuclease core of human Dicer, into the EM map suggests two possible overall molecular architectures for human Dicer. These results offer insights into the structure of Dicer proteins found in multi-cellular organisms and provide a conceptual framework for understanding the initiation of RNAi.
Structure of the Nuclear Envelope (527 – 538)

527/B474
LRMP Is a Nuclear Envelope Protein That Interacts with IP3R.
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The LINC-complex is a protein structure that spans the nuclear envelope (NE) to connect the nucleoskeleton to the cytoskeleton. This complex consists of translumenal associations between inner nuclear membrane SUN-domain proteins and outer nuclear membrane KASH-domain proteins. We propose that lymphocyte restricted membrane protein (LRMP) is a fifth member of the mammalian KASH-family based on its C-terminal KASH-like domain. We can demonstrate that this domain is sufficient for NE targeting and is dependent on association with SUN domain proteins. Unlike conventional KASH family members whose cytoplasmic domains bind to cytoskeletal constituents, LRMP was instead found to interact with inositol triphosphate receptors (IP3R), ER-resident calcium channels. Furthermore, we have characterized the regions of LRMP required for its association with IP3R. Our data suggest a model in which LRMP modulates IP3R at the NE, thus potentially regulating perinuclear cytosolic calcium in lymphocytes.

528/B475 ABSTRACT WITHDRAWN

529/B476
Mechanisms for Active Transport of the Large Sun Protein UNC-84 to the Inner Nuclear Membrane.
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Inner nuclear membrane (INM) proteins must be trafficked from their sight of synthesis, the ER. INM proteins move along continuous membrane domains from the ER to the outer nuclear membrane before crossing the pore membrane to reach the INM. Proteins are retained at the INM by interacting with the nuclear lamina. Three different models have been proposed for the mechanisms of INM trafficking: diffusion-retention and active import using either soluble machinery with a classical nuclear localization signal (cNLS) or a membrane-bound INM-sorting motif (INM-SM). The larger the size of the INM protein, the more difficult it is to cross the nuclear pore membrane. The C. elegans SUN protein UNC-84 is the largest known component of the INM, making it an excellent model to elucidate mechanisms of targeting proteins to the INM. In Vivo deletion analysis suggested that UNC-84 has one membrane spanning domain that separates its 56 kDa N-terminal nucleoplasmic domain from its 64 kDa C-terminal luminal domain. The N-terminus of UNC-84 was necessary and sufficient to target UNC-84 to the nucleus when it was either membrane-bound or soluble. A soluble N-terminal fragment of UNC-84 containing the first 370 amino acids was sufficient for active transport into the nucleus and retention at the nuclear periphery. Further deletion mapping studies revealed that residues 174-370 contained signals that were both sufficient for active import and nuclear envelope retention. Two putative NLSs that resembled classical NLSs were not required for UNC-84 trafficking, but an inner nuclear membrane sorting motif (INM-SM) was required for efficient NE targeting in early embryogenesis. A novel NLS conserved in other SUN proteins was found to be essential for UNC-84 trafficking. These data show for the first time in metazoans that large integral membrane proteins are actively trafficked to the inner nuclear membrane and that the mechanisms used to target SUN proteins to the INM may be conserved.

530/B477
The Reformation of Post-Mitotic Nuclear Envelope by Endoplasmic Reticulum Cisternae Followed by the Insertion of Nuclear Pore Complexes.
During interphase, the Endoplasmic Reticulum (ER) of mammalian cells is made of a continuous network of cisternae and tubules. During mitosis, the ER is mainly reorganized into a network of cisternae, into which the nuclear envelope merges. The disassembly of nuclear pore complexes coincides with the disappearance of the nuclear envelope. How are the nuclear envelope and nuclear pore complexes reformed at the end of mitosis? Using fast live cell 3D-imaging combined with electron microscopy tomographic reconstructions of high pressure freeze/substituted cells, we show that mitotic ER cisternae directly generate the nuclear envelope upon their contact with the surface of the chromosome mass. Formation of nuclear pores is defined here as the sequential recruitment of Nup133 and Nup62, components of the Nup107-160 and Nup62 complexes, respectively. Data obtained by fast live cell imaging show that de novo formation of nuclear pores is strictly constrained to sites already containing nuclear envelope. In contrast, prior to nuclear envelope formation there is no recruitment of Nup133 onto the surface of the chromosome mass. We propose that the assembly of nuclear pore complexes on the nuclear envelope at the end of mitosis is based on an insertion mechanism, probably similar to the process during interphase.

531/B478
Phosphorylation of Nuclear Pore Complex Proteins by ERK Map Kinase Regulates Interaction with Transport Receptors.
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Though many substrates have already been identified for ERK, its roles in regulating various physiological processes suggest that the list is not complete. Recently, we have developed a phosphoproteomic approach to identify putative ERK targets by combining the steroid receptor fusion system, IMAC, 2D-DIGE, and phosphomotif-specific antibodies. As a result, 24 novel targets were identified of which 13 were validated as direct substrates for ERK in vitro. Of these, the nucleoporin Nup50/Npap60 was further verified as an In Vivo ERK substrate by generating phospho-specific antibodies. Nup50 contains an N-terminal domain that binds to importin-α, a central FG repeat domain that binds to importin-β, and a C-terminal Ran binding domain. Interestingly, ERK phosphorylation of the FG repeat region of Nup50 was found to reduce its affinity for importin-β family proteins, importin-β and transportin. In contrast, interaction with importin-α, Ran-GTP, CAS, and Nup153 did not change upon phosphorylation of Nup50 by ERK. Phosphate-affinity polyacrylamide gel electrophoresis using Mn²⁺-Phos-tag followed by Western blotting revealed that ERK stoichiometrically phosphorylates other FG nucleoporins including Nup214, Nup153, Nup98, and Nup62 in intact cells. Of these, phosphorylation of Nup214 and Nup153 by ERK also inhibited their interaction with Importin-β. Furthermore, energy-independent nuclear migration of importin-β and transportin was impaired in ERK-activated, digitonin-permeabilized cells. Finally, RNAi and rescue assays showed that ERK phosphorylation of Nup50 was responsible for this impairment. We propose that ERK may phosphorylate various nucleoporins to regulate functions of importin-β family proteins.

532/B479
Emerin Binding to Lmo7 Inhibits Lmo7 Binding to the Pax3 and MyoD Promoters and Expression of Critical Myoblast Differentiation Genes.
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Mutations in the gene encoding Emerin cause X-linked Emery-Dreifuss muscular dystrophy. Recent evidence suggests the disease phenotype is caused by defective muscle regeneration.
Lmo7 was previously identified as an emerin-binding transcription activator that regulates a number of muscle genes. We report here that Lmo7 regulates C2C12 myoblast differentiation by regulating the expression of critical myoblast proliferation and differentiation genes, including Pax3, Pax7, Myf5 and MyoD. Lmo7 associates with these promoters in myoblasts and binds directly to the promoters of Pax3 and MyoD in vitro. Emerin was previously predicted to inhibit transcription regulator activity by binding transcription regulators at the nuclear envelope and block transcription factor binding to their promoters. Here we directly tested this model and report that emerin binding to Lmo7 inhibits Lmo7 binding to Pax3 and MyoD promoters. We conclude that the physiological interaction between emerin and Lmo7 is critical for myogenic differentiation and muscle regeneration. These data further suggest that loss of emerin in X-EDMD might lead to muscle regeneration defects by causing aberrant temporal activation of myoblast differentiation genes by failing to regulate Lmo7 activity.

Net37, a Nuclear Envelope Transmembrane Protein with Glycosidase Homology, Is Involved in Myoblast Differentiation.
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The nuclear lamina and its associated proteins are important for nuclear structure, chromatin organization, regulation of cell signaling and gene expression. Mutations in several nuclear envelope (NE) proteins have been shown to cause human genetic disorders, including several types of muscular dystrophy. NET37, which was initially identified in our lab, is highly expressed in muscle and up-regulated in differentiated C2C12 cell (Chen et al., 2006). NET37 protein has been shown to be highly concentrated at the NE and lamina-associated (Chen et al., 2006). Here we demonstrate that NET37, a member of glycosidase family 31, is required for myogenic differentiation of C2C12 cells. By protease mapping we show that its glycosidase homology domain is located in the lumen of the NE/ER. When NET37 is depleted from proliferating myoblasts by RNAi, myogenic differentiation is significantly impaired, and there is a concomitant delay in up-regulation of the late myogenic transcription factor myogenin. We expressed silencing-resistant NET37 mutated at a conserved residue in the glycosidase domain and found that this predicted catalytically inactive protein is unable to support myogenesis in cells depleted of wild type NET37. Therefore the enzymatic function of NET37 appears to be important for myogenic differentiation. C2C12 cells depleted of NET37 have reduced activation of Akt after shifting to differentiation medium, and are defective in IGF-II secretion, an autocrine/paracrine factor involved in Akt activation. We also observed that pro-IGF-II co-immunoprecipitates with NET37. Based on our results, we propose that NET37 has a role in IGF-II maturation in the secretory pathway during myoblast differentiation. The localization of NET37 at the NE raises the possibility that it may coordinate myogenic events between the nuclear interior and the ER lumen via transmembrane communication.

Protein Components of the Nuclear Pore Complex [NPC] and Epigenome Have Conserved Domains for Interaction with Functional Non-Coding ncRNA.
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OBJECTIVE: Functional small hairpin ncRNA may address defined homologous helix-nucleating domains shared in epigenetic regulator proteins entangled in growth, metabolic syndromes, vascular, cancer and epigenetic and genetic information indexing of the epigenome. They occur in the extracellular matrix, cytoplasm and the nucleus. Whether or not such ncRNA may be subject to traffic through the NPC escaped consideration, so far. Therefore, as a requisite, protein components of the nucleus [epigenome] and NPC were investigated for interaction domains with such ncRNA. METHODS are described in Ann. N.Y. Acad. Sci. 1137: 316-342, 2008. RESULTS: As marker for the search, functional edited, modified, redox- and metalloregulated small hairpin ncRNA [<200n] as bioaptamers in RNP complexes were isolated from transcriptional RNA output of cells activated by extrinsic [environmental] factors. Some address homologous helix-nucleating
structural proteomic domains, termed K/RxxH [K/R3H], i.e.-t/s/xK/R/q/n/hxxH/y/n/q/e/d/r/kx7-9h/xx7-9h/xx5-20K/R/q/n/e/h- with accessory canonical basic [R/K]n, R/K-zipper, SR/K/RS, EF-hand and/or HxxxH/y/n/q segments. Several components of chromatin formation modulation and NPC were now found which show up such homologous domains highly conserved. Most prominent representatives are of the nucleosomal assembly, histone deacetylase, DNA-C5-methyltransferase, sirtuin, hypoxia-inducible factor, nuclear pore complex, nucleoporin, importin, exportin and nuclear transport factor protein families. CONCLUSIONS: Based on these molecular imprints, the results suggest that functional small hairpin ncRNA participate in functions of the nucleus [epigenome] and the NPC by redox- and metalloregulated interaction with some of their protein components. By this interaction on molecular imprints, they may be subject to traffic or shuttles through the nuclear pore.

535/B482
Two Distinct Pathways of Nuclear Pore Complex Assembly in Higher Eukaryotes.
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In metazoa, the formation of nuclear pore complexes (NPCs) occurs at two different stages during the cell cycle. at the end of mitosis, NPCs are reassembled concomitantly with the reforming nuclear envelope (NE) and in interphase pores form de novo into fully formed NE sheets. The molecular mechanisms and potential differences between post-mitotic and interphase pore assembly are not well understood. Here we show that the RNAi-mediated knockdown of Elys, a nucleoporin that is required for the recruitment of the essential Nup107/160 complex to chromatin, blocks post-mitotic assembly, but is dispensable for interphase assembly in cultured cells. Conversely, depletion of the transmembrane NPC component POM121 specifically inhibits interphase pore formation. Furthermore, POM121 function depends on a functional nuclear localization signal, suggesting that it is required from both sides of the NE. Importantly, pores are located at fusion sites between the inner and outer nuclear membranes, which exhibit high curvature. Nup133, a member of the Nup107/160 complex, has been shown to contain a domain, called ALPS, which exhibits specificity towards highly curved membranes. Strikingly, a single mutation in the ALPS domain, which inhibited membrane-sensing activity of Nup133, resulted in the mislocalization of the Nup107/160 complex to the cytoplasm. Moreover, replacement of Nup133 by the point mutant inhibited new pore insertion during interphase, but had no effect on post-mitotic assembly. In the Nup133 mutant background we also observed at high frequency the appearance of pore intermediates containing POM121 but not the Nup107/160. This suggests that POM121 is present at new pore sites before the Nup107/160 complex. Taken together, our data provide the first evidence for the existence of two distinct NPC assembly pathways in metazoa. Post-mitotic assembly involves ELYS-dependent recruitment of the Nup107/160 complex to chromatin. In contrast, targeting of the Nup107/160 complex to new assembly sites at intact NEs requires POM121 and the ability of Nup133 to sense curved membranes.

536/B483
Direct Actin Binding to A- and B-Type Lamin Tail Domains, and Unique Actin Filament Bundling Activity of the Lamin a Tail In Vitro That Is Disrupted by EDMD-Causing Mutations.
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Nuclear intermediate filament networks formed by A- and B-type lamins are major components of the nucleoskeleton with growing links to human physiology and disease including Emery-Dreifuss muscular dystrophy (EDMD), lipodystrophy, cardiomyopathy, neuropathy, cerebellar disorders and segmental accelerated ‘aging’ syndromes. How lamins interact with other nucleoskeletal components, and the identities of these other components, are open questions. Two previous studies suggested lamins might bind actin. We report that the C-terminal tail domain of human A- and B-type lamins bind directly to purified actin in high-speed pelleting assays. This interaction maps to residues 461-536 within the Ig-fold region of lamin A, which is 54% identical in lamin B1
and defines a conserved Actin Binding site (AB-1). Two EDMD-causing missense mutations (R527P and L530P) in lamin a that are predicted to disrupt the Ig-fold, each reduced F-actin binding by ~66%, whereas the surface-exposed lipodystrophy-causing R482Q mutation had no significant effect. The lamin a tail was unique among all lamin isoforms in having a second actin-binding site (AB-2). This second site was mapped to lamin a tail residues 563-606, based on comparison to the lamin C tail and internal deletions in the lamin a tail that cause Hutchinson-Gilford Progeria Syndrome ($\Delta 35$, $\Delta 50$) or restrictive dermopathy ($\Delta 90$). Supporting the presence of two actin-binding sites, both the precursor and mature lamin a tails (not C or B1 tails) bundled F-actin in vitro. F-actin bundling was reduced 25-40% by the R527P, L530P, $\Delta 35$ and $\Delta 50$ mutations, and was abolished by $\Delta 90$. However the mature lamin a tail unexpectedly both bound, and bundled, F-actin significantly more efficiently than did the prelamin a tail. We therefore suggest that residues 647-664, unique to prelamin A, auto-inhibit binding to actin (and potentially other partners) as a mechanism to regulate its interactions prior to maturation and assembly. These results suggest lamins and actin associate directly in the nucleus, with implications for many pathways including transcription, nuclear export, chromatin remodeling, chromatin movement and nuclear assembly that require lamins, nuclear myosin, and polymerizable actin.

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Nuclear-Cytoskeletal Coupling in Muscular Dystrophies.
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Nuclear-cytoskeletal coupling is essential for force transmission from the extracellular matrix to the nuclear interior and participates in a wide range of physiological processes, ranging from nuclear positioning to cell polarization. The major molecular components involved in nuclear-cytoskeletal coupling known to date are SUN proteins and nesprins, which form the LINC complex, physically linking the nucleus to the cytoskeleton. However, it remains unknown whether the LINC complex is the major physical component responsible for transmitting intracellular forces to the nuclear interior. Objective and Methods: to test the hypothesis that the LINC complex is the major contributor in nuclear-cytoskeletal force transmission, we used a microneedle manipulation assay to quantify induced cytoskeletal and nuclear deformations after localized cytoskeletal force application in cells in which the LINC complex had been disrupted by dominant negative mutants and in wild-type controls. To explore whether altered nuclear-cytoskeletal force transmission may affect cellular polarization, we evaluated centrosome positioning in a wound healing assay. Results: Cytoskeletal strain transmission across the nucleus was significantly reduced in cells with a disrupted LINC complex compared to wild-type and mock control cells. Interestingly, similar results were found in cells lacking the nuclear envelope proteins lamin A/C. Induced nuclear strain, near the force application site (cis) and across from it (trans), was significantly reduced in LINC disrupted cells, suggesting a loss of force transmission from the cytoskeleton to the nucleus. In lamin A/C-null cells, induced nuclear strain across from the force application site, but not near the application site, was reduced compared to wild-type cells, providing evidence for a dissipation of forces across the softer nucleus. Preliminary results from the wound healing assay showed an impaired centrosome polarization in the LINC disrupted cells, which indicates a disruption in nuclear-cytoskeletal force transmission. Conclusions: Disruption of the LINC complex results in impaired force transmission from the cytoskeleton to the nucleus and may contribute to defects in cellular functions.

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Architectural Nucleoporins Nup157/Nup170 and Nup133: A Structural Perspective on Ancestral Elaboration within the Nuclear Pore Complex.
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The nuclear pore complex (NPC) is one of the largest protein assemblies in the eukaryotic cell; it forms the exclusive gateway to the nucleus. In the NPC, the stable, ~15-20 MDa scaffold ring is built with eight-fold symmetry from two multiprotein subcomplexes. These subcomplexes are assembled from large architectural nucleoporins. Among these nucleoporins are Nup120 and four ancestral coatamer element (ACE1) nucleoporins. We present here crystal structures of two further large architectural units, γNup170 and hNup107-hNup133. Nup170 and Nup133 have conserved domain arrangements and, as we now show, conserved tertiary structures, distinct from all other nucleoporins and vesicle coat proteins. This conservation suggests that Nup170 and Nup133 derive from a common ancestor. These units, constituted, along with the ACE1 nucleoporins, the major alpha-helical building blocks of the NPC scaffold and define its branched, lattice-like architecture, similar to vesicle coats like COPII. We hypothesize that the extant NPC evolved early in eukaryotic evolution, by gene duplication and diversification, from a simpler structure that had several identical copies of a few ancestral elements.

**Growth Factors and Receptor (539 – 552)**

**539/B486**

**Passive Diffusion of Naltrexone into Human and Animal Cells and Upregulation of Cell Proliferation.**

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Naltrexone (NTX) is a potent opioid antagonist that promotes cell proliferation by upregulating DNA synthesis through displacement of the tonically active inhibitory peptide, opioid growth factor (OGF), from its receptor (OGFr). To investigate how NTX enters cells, NTX was fluorescently labeled (FNTX, 1-(N)-fluoresceinyl naltrexone thiosemicarbazone) to study its uptake by living cultured cells. When human head and neck squamous cell carcinoma cell line SCC-1 was incubated with FNTX for as little as 1 min, cells displayed nuclear and cytoplasmic staining of FNTX as determined by fluorescent deconvolution microscopy, with enrichment of fluorescent signal in the nucleus and nucleolus. The same temporal-spatial distribution of FNTX was detected in a human pancreatic cancer cell line (MIA PaCa-2), African green monkey kidney cell line COS-7, and human mesenchymal stem cells (hMSCs). FNTX remained in cells for as long as 48 h. FNTX was internalized in SCC-1 cells when incubation occurred at 4°C, with signal being comparable to that recorded at 37°C. A 100-fold excess of NTX or a variety of other opioid ligands did not alter the temporal-spatial distribution of FNTX. Neither fluorescein labeled dextran nor fluorescein alone entered the cells. To study the effect of FNTX on DNA synthesis, cells incubated with FNTX at concentrations ranging from 10⁻⁵ to 10⁻⁸ M had a BrdU index that was 39-82% greater than for vehicle treated cells, and was comparable to that of unlabeled NTX (37-70%). Taken together, these results suggested that NTX enters cells by passive diffusion in a non-saturable manner.

**540/B487**

**Nuclear Import of the Opioid Growth Factor Receptor (OGFr) to Regulate Cell Proliferation Is Dependent on Karyopherin β and Ran.**

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The Opioid Growth Factor (OGF) Receptor (OGFr) is an integral component of the OGF-OGFr axis which regulates cell proliferation in normal and neoplastic tissues and cells. Regulation of cell proliferation by the OGF-OGFr axis is dependent on translocation of OGFr from the cytoplasm into the nucleus. Using the human squamous cell carcinoma of the head and neck cell line SCC-1, and OGFr fused to enhanced GFP (OGFr-eGFP), we examined the nuclear import of OGFr with particular focus on karyopherin β₁, karyopherin α, and Ran. Transfection of SCC-1 cells with karyopherin β₁ siRNA down-regulated protein expression of karyopherin β₁ by 54%, and prevented transport of ~50% of OGFr-eGFP into the nucleus. Transfection of SCC-1 cells
with Ran specific siRNA inhibited the nuclear import of OGFr-eGFP by ~55%. Downregulation of karyopherin proteins α1, α2, α3, α4 or α6, which serve as adaptor proteins for karyopherin β1, blocked less than 4% of OGFr-eGFP transport into the nucleus, suggesting that they do not play an integral role in OGFr transport. To determine whether karyopherin α/β or Ran is(are) a requirement for normal function of the OGF-OGFr axis in cell proliferation, DNA synthesis was monitored using BrdU-labeled cells transfected with karyopherin α, karyopherin β, Ran siRNAs, or scrambled siRNA. Scrambled siRNA transfected cells, as well as karyopherin α groups, with OGFr-eGFP staining had 50% BrdU-positive cells, whereas cells transfected with karyopherin β1 or Ran siRNA had a 70% labeling index. These data indicate that disabling of karyopherin β1 or Ran using siRNA upregulates cell proliferation by blocking the tonically active inhibitory pathway of OGF-OGFr. Therefore, the regulation of cell proliferation by the OGF-OGFr axis is dependent on the integrity of karyopherin β1 and Ran.

541/B488
‘Rotation/Twist’ Model for the Mechanism of the EGF/ErbB Receptor Activation.
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The epidermal growth factor (EGF)/ErbB receptor family consists of four members, and plays pivotal roles in the development of organisms ranging from worms to humans. Furthermore, aberrant activation of the receptors is frequently implicated in a variety of human cancers. Ligand-induced dimerization is proposed as a molecular mechanism underlying the activation of all the growth factor receptor tyrosine kinases including the ErbB receptor family. Before ligand binding, however, it remains controversial whether the receptors have a monomeric or dimeric structure. We have shown that most, if not all, molecules on the cell surface of all the ErbB receptors have preformed, yet inactive, homo- and heterodimeric structures in living cells by using chemical cross-linking, cysteine disulfide bridge formation, sucrose density gradient centrifugation, Förster resonance energy transfer (FRET), fluorescence cross-correlation spectroscopy (FCCS), and fluorescent protein and luciferase fragment complementations. The spontaneous dimerization occurs in endoplasmic reticulum, where ligands are unlikely to be available, before newly synthesized receptors reach the cell surface. Together with the transmembrane and extracellular juxtamembrane domains, the cytoplasmic domains seem to be mainly responsible for the spontaneous inactive dimer formation. Based on crystal structures of the cytoplasmic domains recently determined, we have artificially made several mutations of residues residing in the interface between the homodimeric structure of the cytoplasmic kinase domains, and have found that these mutations induce spontaneously the receptor autophosphorylation. These results indicate that the receptor exist as a preformed dimer mainly through interaction between the kinase domains. Based on current and previous results, we will propose a ‘rotation/twist’ model for the molecular mechanism of the ErbB receptor activation by ligand, in which ligand binding to the extracellular domains of the receptor dimer induces rotation/twist of the receptor’s transmembrane domains, and rearrange the intracellular kinase domains for activation.

542/B489
Young and Aged Human Muscle Satellite Cells Show Differential Expression of S100b Protein and Rage.
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During aging, skeletal muscles show reduced functional capacity due to loss of the regenerative ability of satellite cells (SCs), the quiescent stem cells located beneath the basal lamina surrounding each myofiber. Both age-related extrinsic factors and age-related intrinsic properties of SCs appear to contribute to SC efficiency (Aging Cell 7 (2008) 590-8). In the present work we analyzed several parameters of SCs derived from biopsies of Vastus Lateralis muscle from healthy non-trained young and aged humans (male and female, divided into young [mean age 31.0 ± 5.35 years; n=7] and aged [mean age 76.44 ± 6.12 years; n=9]). Compared to young SCs,
Aged SCs showed impaired differentiation, i.e. reduced myotube formation and reduced expression of myogenin and myosin heavy chain when cultured in differentiation medium (DM), and exhibited the following: i) reduced proliferation; ii) higher expression levels of S100B, a Ca2+-binding protein and negative regulator of myoblast differentiation (submitted for publication); iii) undetectable levels of full-length RAGE (receptor for advanced glycation end products), a multiligand receptor of the immunoglobulin superfamily, the engagement of which enhances myoblast differentiation (Mol Cell Biol 24 (2004) 4880-4894; J Biol Chem 281 (2006) 8242-8253), and presence of a truncated form of RAGE in growth medium (GM) the amount of which decreased in DM in parallel with appearance of full-length RAGE; and iv) lower expression levels of the transcription factors, MyoD and Pax7, in both GM and DM. Also, transient transfection of young SCs with S100B expression vector resulted in reduced differentiation compared to controls (i.e., acquisition of an aged phenotype), while either transfection of aged SCs with full-length RAGE expression vector or knocking down S100B by siRNA resulted in enhanced differentiation (i.e., acquisition of a young phenotype). These data point to an important role for intrinsic factors (e.g., MyoD, Pax7, S100B and RAGE) in defective SC function in aged skeletal muscles.

543/B490
Vascular Endothelial Growth Factor Is Involved in Bone Bridge Formation at the Physeal Injury Site.
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Physeal injuries can initiate the formation of reversible bone-bridges resulting in bone length discrepancy, axis deviation or joint deformity. We hypothesized that vascular endothelial growth factor (VEGF) and its receptors R1 and R2 are involved in the vascularization process leading to bone bridge formation at the site of physeal injury. 35 male Sprague Dawley rats (4 weeks old, 100g) were subjected to a unilateral drill injury through the proximal tibial physis. After euthanasia on days 1, 3, 7, 14, 28, 42 and 82 both tibiae were extracted. Tissue samples of the growth plates were processed for immunohistochemical staining of VEGF and VEGFR1 and -R2 and Hematoxylin and Eosin staining. The contra-lateral intact growth plate samples served as controls. Histological staining displayed a hematoma on days 1 and 3 containing displaced bone fragments at the site of physeal injury. on day 7, reorganization of these fragments was observed prior to bone bridge formation seen on day 14. By day 82, this bone bridge was replaced by lamellar bone. Immunohistochemical staining showed clear positivity for VEGF, VEGF-R1 and VEGF-R2 of the displaced bone fragments on days 1 to 7. While the bone bridge remained VEGF positive, with descending intensity from proximal to distal within the bone bridge on day 82, VEGF-R1 and -R2 were initially absent. VEGF-R2 was positive in the early lamellar bone formation on day 42. Displaced chondrocytes bordering the physis at the injury site were VEGF, VEGF-R1 and -R2 positive throughout the days, showing maximums of R1 on day 14 and of R2 on days 14 to 42. These data suggest that bone fragments from the physeal injury are interacting with cells bordering the physeal injury site and together initiate bone bridge formation via expression of the angiogenic growth factor VEGF, and its receptors R1 and R2.

544/B491
Thrombomodulin Domain Promotes Angiogenesis through FGFR1 Pathway.
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Thrombomodulin (TM) is a type I glycoprotein on endothelium, which serves as a receptor for thrombin. TM/thrombin complex catalytically activates protein C and thus triggers anticoagulation cascade with protein S. The recombinant EGF-like domain plus serine/threonine rich domain of TM (rTMD23) has been shown to promote angiogenesis In Vitro and in vivo. Concurrently, activated protein C has also been demonstrated to induce angiogenic responses. However, the
mechanism of rTMD23 on inducing angiogenesis is unclear and whether the angiogenic activity of rTMD23 is protein C dependent or not is obscure. rTMD23 proteins lacking protein C activation ability were prepared by site-directed mutagenesis. All three mutant proteins without protein C activation activity showed similar activity as rTMD23 in promoting endothelial cells proliferation, migration and tubular morphogenesis in vitro. In addition, we demonstrated that rTMD23 interacted with heparin Sepharose, syndecan 4 and type I fibroblast growth factor receptor (FGFR-I). rTMD23 effectively induced phosphorylation of tyrosine residue 653/654 of FGFR-I in human umbilical vein endothelial cells and in exogenously FGFR-1 over-expressed human embryonic kidney 293 cells. The activation of FGFR-I induced by rTMD23 was attenuated by PD173074, a specific inhibitor to FGFR-I. Furthermore, the rTMD23-induced angiogenic responses in vitro, such as cell proliferation, migration and tubular morphogenesis in human umbilical vein endothelial cells, were significantly inhibited by PD173074. In conclusion, rTMD23 interacts with heparan sulfate proteoglycans thereby activating FGFR-I and consequently turns on the angiogenic reaction through a protein C independent pathway.

545/B492
The Bile Acid, Taurocholate, Prevents Biliary Damage Induced by In Vitro Hypoxia by Changes in the Expression of Angiogenic Factors in Cholangiocytes.

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The function of the biliary tree is linked to the vascular supply sustained by the peribiliary vascular plexus, and its circulating angiogenic factors such as VEGF and angiopoietin (Ang). VEGF regulates the recovery phase of reperfusion injury. We have shown that: (i) taurocholic acid (TC) prevents bile duct damage by changing VEGF expression; and (ii) enhanced expression of cholangiocyte angiogenic factors regulates biliary functions in an In Vivo model of ischemia/reperfusion injury. Using an In Vitro system, we aimed: (i) to elucidate if reoxygenation of ischemic cholangiocytes regulate changes in the expression of angiogenic factors; and (ii) if TC protects cholangiocytes against ischemic damage. Methods: Rat cultured cholangiocytes (NRICC) were maintained under standard conditions in normoxic atmosphere of 21% O2, 74% N2 and 5% CO2 or under a hypoxic environment of 5% O2, 90% N2 and 5% CO2 for 4 hours and transferred to normal conditions for different times (1-4 hours) in the absence/presence of TC (40 μM). Then, we collected NRICC protein, mRNA and supernatants to measure changes in: (i) proliferation by PCNA immunoblots, (ii) apoptosis by Bax immunoblots, (iii) expression of VEGF-A/-C, VEGFR-2/-3, Ang1/2 and Tie1/2 by immunoblots and real-time PCR, and (iv) VEGF secretion. Results: Under hypoxic conditions, there was decreased NRICC growth, and increased cholangiocyte apoptosis and expression of VEGF-A/-C, VEGFR-2/-3, Ang1/2 and Tie1/2 coupled with enhanced NRICC VEGF secretion. Recovery of the normoxic conditions led to restoration of cholangiocyte proliferation and the expression of cholangiocyte angiogenic factors in a time-dependent manner. TC protected against biliary damage and upregulation of angiogenic protein expression under the hypoxic conditions. Conclusion: We hypothesized that: (i) hypoxia increases damage of cholangiocytes and alters the expression of biliary angiogenic factors and VEGF secretion in NRICC likely due to an autocrine compensatory mechanism; and (ii) TC prevents biliary damage in pathological conditions of lowered oxygen supply. Altered expression of biliary angiogenic factors through modulation of oxygen content may be important in the management of liver diseases.

546/B493
The Role of Rin1 Mutants in EGFR Internalization and Cell Signaling.
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The internalization and further activation of the epidermal growth factor receptor (EGFR) is required for several intracellular processes, including membrane transport, fusion, and signaling. Several intracellular molecules, including Ras Interference 1 (Rin1), have been shown to play a key role during internalization and signaling of the EGFR. In this study, we examined the role of key conserved amino acids in the Vps9 domain of Rin1 on the EGFR internalization and cell signaling. In addition, we investigated the role of these conserved residues in the fusion reaction. Six of the ten mutants were unable to stimulate and rescue the fusion reaction. Two of them, Rin1: D537A and Rin1: Y561F, drastically inhibited the fusion reaction. Furthermore, these two mutants, but not Rin1: wild type, failed to block the Ras/MAP kinase cascade activated by EGF. Taken together, these data suggest key amino acids in the Vps9 domain of Rin1 play an important role on the regulation of EGFR trafficking and signaling.

547/B494

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An important component of the vascular wall repair process is the recruitment of smooth muscle cells (SMC) from the existing contractile coat. Phenotypic modulation of these SMCs enables them to proliferate and migrate into the vessel intima. Despite its importance in vessel repair, this plasticity of SMCs can promote both the pathogenesis of atherosclerosis as well as neointimal formation following revascularization-induced injury. Vascular growth factors contribute significantly to the migratory and proliferative component of the response to injury. IGF-1 is one such growth factor which elicits a response via its receptor, the IGF-1R, a classical tyrosine kinase receptor. However, it has also been reported to couple to a heterotrimeric G protein in rat fibroblasts. The objective of this study was to investigate the structural aspects of the association between the IGR-1R and heterotrimeric G proteins in SMCs, as well as the contribution of this pathway to cellular responses. In a porcine primary smooth muscle cell culture model, the IGF-1R was found coupled to a Gβ subunit in the quiescent state. Cellular activation with IGF-1 decreased this interaction by nearly 60%, while inhibition of the IGF-1R tyrosine kinase with AG1024 returned G protein coupling to basal levels. A Gβγ modulator, Gallein, was used as a tool to determine the role of this subunit in the response. It was found that phosphorylation, and thus activation, of p42/44 MAPK was only inhibited by 50% by AG1024, but returned to basal levels when treated with Gallein. The data thus far support the conclusion that IGF-1 elicits its response on vascular SMCs at least partially via a non-classical G protein-coupled receptor, although activation of the tyrosine kinase receptor also plays a role. In addition, at least one of the phenomena responsible for revascularization injury, proliferation, is modulated via the Gβγ subunit of the pathway.

548/B495
Sciatic Nerve Conditioned Medium (CM) Increases NG2, Nestin and Galectocerebroside Positive Cells and Reduces the Complexity of Dendritic Process.

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In the Peripheral (PNS) and Central (CNS) Nervous Systems there are different kinds of glial cells responsible for releasing factors that affect the regeneration process after injury; being the regeneration in the PNS more efficient than in the CNS. In previous works, we had demonstrated that factors released by sciatic nerves in culture, increased the number of type 2 astrocyte, promoted the differentiation of type 1 astrocytes and decreased the neuron population in primary hippocampal cultures. Additionally, we observed a different population of cells in the culture that were negative for astrocyte and neuronal markers. To evaluate the proportion of mature
oligodendrocytes, oligodendrocyte precursors and neural precursors, hippocampal cultures were immunostained with antibodies against galactocerebroside (GalC), chondroitin sulfate proteoglycan (NG2) and nestin, respectively. We found that CM increased GalC positive cells from 11.15% (2 days) to 23.63% (15 days). CM also increased NG2 positive cells from 5.17% (2 days) to 26.79% (7 days). In contrast, in the control condition the proportion of GalC positive cells were similar at all days (5.06%), while NG2 positive cells were absent at 2 and 5 days but started to show at 7 days (10.19%). Nestin positive cells were similar at 2d in both condition (Control: 11.43%, CM: 11.2%) but at 7d there was an increase on nestin positive cells (CM: 33.85%, Control: 16.32%). On the other hand, we investigated if the CM had any effect on the neuronal morphology. Immunostaining with MAP2 and SMI35 antibodies showed that the CM decreased the complexity of the dendritic processes compared with the control condition. These and previous results suggest that CM strongly favors glial cells, whereas decreasing both number and neurons complexity.

549/B496
Hyper-Activation of STAT5a Is Associated with LEF-1 Down Regulation and Leukemic Transformation in Patients with Severe Congenital Neutropenia.
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Severe Congenital Neutropenia (CN) is a preleukemic syndrome characterized by maturation arrest of granulocytes in bone marrow at the promyelocytic stage. Cumulative incidence of developing AML/MDS in CN is 20%. Since AML/MDS are not observed in cyclic or idiopathic neutropenia patients treated with G-CSF, an underlying defect down stream of G-CSF signaling rather than G-CSF therapy per se predisposes to malignant transformation in CN. Also, though G-CSF and G-CSFR levels are considerably elevated in CN, high pharmacological doses of G-CSF (100-1000 times higher than the physiological levels) are required to increase neutrophile counts in CN. Therefore, G-CSF signaling is severely affected in CN patients. STAT5 is a downstream effector of G-CSF signaling pathway and is found to be activated in AML. LEF-1 transcription factor and its target gene C/EBPα are severely abrogated in CN. Therefore, we investigated the effects of G-CSF on the phosphorylation status of STAT5a in CN and if it leads to down modulation of LEF-1 expression and functions. We found that G-CSF stimulation resulted in a significantly higher phosphorylation of STAT5a in CD34+ cells of CN patients as compared to healthy volunteers. Transduction of constitutive active STAT5a (STAT5a 1*6) in CD34+ cells of healthy individuals resulted in severe downregulation of LEF1 expression in a dose dependent manner. A screen of 10 kb upstream region of LEF1 gene revealed two putative STAT5 binding sites, confirmed by ChIP assay and No shift assay using nuclear extracts of CD34+ cells and U937 respectively. We found enhanced and prolonged STAT5a binding to the LEF-1 promoter in G-CSF treated CD34+ cells from CN patients, as compared to healthy individuals. Additionally, transfection of CD34+ cells with LEF-1 cDNA resulted in elevation of LEF-1 promoter activity and co-transfection with STAT5a 1*6 significantly disrupted LEF1-dependent activation of LEF-1 promoter. Moreover STAT5a 1*6 severely abrogated the LEF1 dependent regulation of C/EBPα gene promoter. Taken together phosphorylation of STAT5 is enhanced in hematopoietic progenitors of CN which subsequently down regulates LEF1 and may contribute to the malignant transformation of myelopoiesis in CN.

550/B497
Interaction of Growth Hormone Receptor (GHR) and Jak2: A Model System to Study GHR Signaling.
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The Growth Hormone Receptor (GHR) is a single membrane spanning protein of 620 aa, that belongs to the class I cytokine receptor superfamily. Upon binding of Growth Hormone (GH) to the dimeric receptor at the plasma membrane, Janus kinase 2 (Jak2)/STAT5b and downstream signalling pathways are activated via reciprocal phosphorylation, which regulate somatic growth,
substrate metabolism and body composition. The number of GHRs at the cell surface determines GH sensitivity of the cells and needs, therefore, to be tightly regulated. In severe diseases, like cancer and AIDS, the number of GHRs decreases and the body becomes GH insensitive, leading to muscle wasting (cachexia). Understanding GHR endocytosis and degradation will provide new insights how to increase the GH sensitivity of cells, and increase our capabilities to fight serious diseases. Members of the Jak2 kinase family initiate the majority of downstream signalling events of the cytokine family of receptors. Although the diversity of cytokine receptors is large, the prevailing principle is that Jak kinases act as twins: Two similar or different Jaks bind to two cytosolic tails of the homo- or heteromeric cytokine receptor complex and start their signalling activity upon a conformational change in the receptor complex, induced by their cognate cytokine. We have expressed growth hormone receptor tails together with Jak2 in mammalian cells to study the molecular principles of Jak/Stat signalling. Cytosolic tails of the GHR provided with leucine zippers were stabilized if co-expressed with Jak2. This system provides a good model to study early events in growth hormone receptor signalling and the interaction between the GHR and its binding proteins.

551/B498
Detection of Intracellular and Secreted Neurturin.
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Neurturin (NRTN), a member of GDNF family ligands, is a neurotrophic growth factor that regulates the development and maintenance of neuronal populations in the brain and in the periphery. NRTN has functions outside the nervous system too. NRTN is a secreted protein, which is synthesized in a form of precursor, pre-pro-NRTN. NRTN is a potential therapeutic agent for neurodegenerative diseases as Parkinson's disease. Because of the neurotrophic effects of NRTN, it is important to study its poorly known cell biology. Our aims are to detect mouse nrtn intracellularly and to study nrtn protein secretion in non-neuronal cell lines. The tested tissues included kidney, ovary, adrenal medulla, cervix, retina, skeletal muscle and glioma. Second aim is to detect human NRTN mRNA from neuronal and non-neuronal tissues and identify possible splice variants. Non-neuronal cells were first overexpressed with wt mouse nrtn. Intracellular and secreted nrtn was detected in western blotting (WB). The specificity of anti-nrtn antibodies was analysed in WB and immunocytochemistry. Because of the poor quality of the available anti-nrtn antibodies, mouse nrtn was cloned with HA- and FLAG-tags. Tagged nrtn proteins were detected in WB by anti-HA- and anti-FLAG-antibodies. The biological activities of tagged nrtn proteins were confirmed in neurite outgrowth assay in PC6 cells. Human NRTN was detected by reverse transcriptase-PCR from selected tissues, sequenced and cloned into mammalian expression vectors. Our preliminary results suggests that mouse wt nrtn and tagged nrtn proteins are translated and can be detected intracellularly but are not secreted or poorly secreted to the growth medium. Secretion may require a cell specific stimulation, for example a hormonal or electronic factor to induce nrtn secretion. Human NRTN mRNA was detected from fetal and adult brain tissues and from several non-neuronal tissues. One transcript was identified and cloned.

552/B499
TBC1D3, a Hominoid-Specific Protein, Modulates Insulin Signaling by Interacting with Cul7 and Delaying Insulin-Dependent Phosphorylation and Degradation of IRS-1.
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TBC1D3 is a hominoid-specific gene. Recent work showed that TBC1D3 expression enhances the signaling properties of growth factor receptors resulting in Ras activation enhanced cell proliferation (J Biol Chem 283:13233, 2008). We carried out a yeast two-hybrid screen using TBC1D3 as bait and identified Cul7, a Cullin family ubiquitin E3 ligase, as a TBC1D3-interacting protein. TBC1D3-Cul7 interaction was confirmed by immunoprecipitation and In Vitro transcription/translation experiments. Cul7 is reported to mediate the degradation of the insulin
receptor adapter, IRS-1 (Mol Cell 30:403, 1008). We found that expression of TBC1D3 suppressed degradation of IRS-1 and increased Akt signaling pathways in response to insulin treatment. Phosphorylation of IRS-1 at key residues, responsible for Cul7-dependent IRS-1 degradation, was suppressed by TBC1D3 expression. Moreover, TBC1D3 formed a complex with Cul7-FBXW8- and IRS-1 in cells stimulated with insulin. Lastly, insulin stimulation led to the degradation of TBC1D3 suggesting a negative modulatory feedback loop. These data suggest that TBC1D3 provides a regulatory mechanism in humans to control insulin signaling via the phosphorylation and degradation of IRS-1.

Signal Transduction I (553 – 576)

553/B500
Identification of Cellular Targets of Naringenin via Restriction Enzyme Mediated Integration in Dictyostelium.
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Naringenin, a flavanone compound highly enriched in grapefruits, has been identified as an inhibitor of cell proliferation; and thus has the potential to act as an anti-tumorigenic agent. At this time, the mechanism by which naringenin is able to suppress cell growth is unclear since this compound appears to have multiple cellular targets including cytochrome P450 enzymes, the PI3 kinase pathway, glucose uptake pathways, among others. Studies from our lab using Dictyostelium as a model system have revealed that naringenin is a potent inhibitor of eukaryotic cell motility, as well as cell proliferation. However, a common cellular target for naringenin that is shared among different cell types and in varying cellular contexts has eluded identification. As a means of identifying such targets, we used restriction enzyme mediated integration (REMI) to create a library of Dictyostelium mutants that were then screened for resistance to naringenin-induced suppression of cell proliferation. Such mutants are of interest since they are missing some factor (presumably the product of a gene disrupted by REMI) that is necessary for the bioactivity of naringenin. To this end, REMI mutants were generated by electroporating Dictyostelium AX2 cells with BamHI-linearized pBSR-Δ-Bam plasmid, along with 20 U of BamHI enzyme, and then selecting for REMI mutants in HL5 culture medium containing 10 μg/ml blasticidin. We then isolated four naringenin-resistant REMI mutants (of greater than 200 screened) that grow normally in the presence of 300 μM naringenin. These results indicate that the REMI based approach in Dictyostelium is a viable means for identifying genes that required for the bioactivity of naringenin. We are continuing to screen more REMI mutants and are in the process of pulling out and identifying the genes that have been disrupted in the four naringenin-resistant mutants.

554/B501
Quantitative Spatiotemporal Analysis of K-Ras Activation Using Live Cell Redistribution Assays.
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Activating mutations in the Ras protein occur in 33% of human cancers with mutations in the K-Ras isoform being most prevalent. Ras signals through multiple downstream effectors, including PI3K, Raf and RalGEF, which bind to activated Ras, triggering downstream signaling and tumorigenesis. K-Ras is anchored to the plasma membrane (PM) by virtue of a C-terminal farnesy1 group and polybasic region. Activated, GTP bound K-Ras causes c-Raf to redistribute from the cytoplasm to the PM whereupon downstream MAP Kinase signaling is initiated. Assays that measure the activation of K-Ras have relied on PM targeting of the Ras-binding-domain of c-Raf or other Ras effectors. We show that full-length c-Raf and RalGDS can be visualized targeting to the PM in the presence of RasGTP. Fluorescent protein fusions of Ras and effectors were imaged in live cells using confocal microscopy. We have developed an automated image analysis procedure to measure the ratio of PM:cytoplasm localized effector and thus calculate the
percentage of redistribution events per cell. Our quantitative redistribution assay can distinguish changes in Ras activation conferred by activating mutations in the Ras proteins compared with wild-type Ras. Furthermore, the time-course of wild-type K-Ras activation upon stimulation with epidermal growth factor can be visualized and quantified. We validate the assay by demonstrating that a dominant-negative K-Ras S17N mutant fails to cause localization of effectors to the plasma membrane. The assay and analysis we present are valuable tools to identify molecules that are specific in disrupting the interaction of Ras and effectors in the cellular context and that may thus have therapeutic potential.

555/B502

CXCL1 and CXCL8 Induce Morphological Changes in SKMEL 28 and 1205 Lu Melanoma Cells.

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The chemokines CXCL1 and CXCL8 are released by melanoma cells and potentially participate in melanoma progression. This study was designed to determine whether CXCL1 and CXCL8 significantly alter melanoma cell morphology or β-catenin localization. Images of fluorescent antibody-tagged cells were obtained with a Zeiss Axiovert 200M microscope with or without 24h treatment with these chemokines. CXCL1 and/or CXCL8 treatment led to an elongated (1205Lu cell line) or stellate (SKMEL 28 cell line) morphology compared to control. CXCL8 treatment caused a decreased presence of β-catenin at cell-cell junctions as well as a decrease in the number of cell-cell junctions. In both cell lines, regardless of treatment, β-catenin localized on the same side (leading edge) of the nucleus as the Golgi and mitochondria but did not strictly co-localize with either organelle. Control cells in both lines stained with wheat germ agglutinin conjugates, revealed the unexpected presence of glycosylated, branching, extremely thin and long (less than 1 μm in diameter and up to 500 μm long) filopodia-like structures extending from the cell membranes. The branching occurred at blebbed, dot-like regions along the structures as extensions continued from these regions at differing angles. CXCL1 and CXCL8 treatment increased the number of these filopodia-like structures. The structures, regardless of treatment condition or cell line, stained for actin, cytosol, β-catenin, focal adhesion kinase, E-cadherin (low levels), N-cadherin, β1 integrin, β3 integrin (localized in blebbed regions of the structures), as well as CXCL1 and CXCL8 receptors CXCR1 and CXCR2. When CXCR1 and/or CXCR2 were blocked in SKMEL 28 cells (1205Lu yet to be explored), CXCR1 was revealed to be vital in mediating CXCL8’s effect on morphology while CXCR2 was vital in mediating CXCL1’s effect on morphology. Blockade of both chemokine receptors eliminated CXCL1’s and CXCL8’s previously seen increase in the number of filopodia-like structures. We conclude that CXCL1 and CXCL8 have the potential to affect melanoma progression by inducing changes in cell morphology and the formation of filopodia-like structures, and thus, possibly altering intercellular communication.

556/B503

The E1A-Associated p400 Protein Modulates Cell Fate Decisions through the Regulation of ROS Homeostasis.

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The p400 E1A-associated protein, which mediates H2A.Z incorporation at specific promoters, plays a major role in cell fate decisions control: it promotes cell cycle progression and inhibits induction of apoptosis or senescence. Here, we show that siRNA-mediated depletion of p400 induces an oxidative stress transcriptional signature. Moreover, it leads to an increase in intracellular ROS levels and to the appearance of DNA damage and γH2AX foci, indicating that p400 maintains oxidative stress below a threshold at which DNA damage and DNA damage signalling occur. Suppression of these DNA damage response pathways using a siRNA against ATM inhibits the effects of p400 on cell cycle progression, apoptosis induction or senescence, demonstrating the importance of ATM-dependent DDR pathways in cell fates control by p400. Finally, we identify HSP70 as an important target of p400 for ROS modulation since we show that the HSP70-encoding gene promoter is directly activated by p400-mediated H2A.Z incorporation.
and since we can partially restore normal ROS levels in p400-depleted cells by preventing HSP70 decrease. Altogether, our results uncover an unexpected link between p400 and ROS metabolism and allow us to decipher the molecular mechanisms largely responsible for cell proliferation control by p400.

557/B504
The Role of Phosphoinositides as Co-Activators of p21-Activated Kinase 1 (Pak1).
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The Rho-family GTPases Cdc42 and Rac1 have been implicated in signaling pathways that contribute to cancer via the activation of a diverse array of downstream effector proteins. One important effector of these Rho GTPases is the serine/threonine kinase p21-activated kinase 1 (Pak1), whose activity has been implicated in tumorigenesis and metastasis. In its inactive state, Pak1 adopts an autoinhibited conformation that is relieved upon binding to Rac1 and Cdc42, although whether this is the sole mechanism of activation is currently unknown. We have obtained evidence that the phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP2) is capable of activating Pak1 in a manner that is highly cooperative with Rac1/Cdc42. We have demonstrated that Pak1 binds directly to PIP2 and that this interaction requires a positively-charged basic region in Pak1 adjacent to the N-terminal GTPase binding domain. In cytoplasmic extracts from Xenopus laevis oocytes, PIP2-mediated activation of Pak1 is dependent on this stretch of N-terminal basic residues. In mammalian cells, growth factor-stimulated activation of Pak1 requires these same basic residues as does the localization of active Pak1 to PIP2-enriched plasma membrane ruffles. Since both PIP2 and the active GTP-bound forms of Cdc42/Rac1 are localized to the plasma membrane, we hypothesize that Pak1 functions as a coincidence detector, whose full activation depends upon Rho GTPases present in phosphoinositide-rich membrane contexts. As the distribution of various phosphoinositides within the cell is relatively restricted, this mechanism may contribute, in part, to GTPase effector selectivity, ensuring that only a subset of Rac1/Cdc42 effectors are activated in a spatially and temporally appropriate manner. These results also suggest that pathologically elevated levels of PIP2 might contribute to the Pak1 hyperactivity associated with certain human cancers.

558/B505
The Adaptor Protein EBP50 Cooperates with EGFR to Control Biliary Cancer Cell Scattering and Migration.
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The adaptor protein EBP50 (Ezrin-radixin-moesin Binding Phosphoprotein 50) binds membrane receptors including Epidermal Growth Factor Receptor (EGFR). EBP50 and EGFR are both expressed in the biliary epithelium. While EGFR participates in biliary carcinogenesis, implication of EBP50 in this cancer is unknown. The aim of the study was to determine the potential links between EBP50 and EGFR in human biliary carcinoma. Methods: In Vitro studies were performed in the human biliary epithelial cell line, Mz-ChA-1, that retains epithelial phenotype and endogenous expression of EBP50 and EGFR. The role of EBP50 was examined using siRNA. Proliferation was analyzed by MTT test, PCNA expression and Ki67 immunolabeling. Cell scattering and migration were evaluated using E-cadherin immunostaining and Boyden chamber, respectively. for In Vivo study, 111 patients who underwent surgical resection for cholangiocarcinoma were analyzed retrospectively. Immunohistochemistry on tissue microarrays was performed to assess EBP50 and EGFR expression. Results: EGF activates EGFR and the signaling pathways, ERK1/2, STAT3 and Akt, in Mz-ChA-1 cancer cells. Inhibition of EBP50 in EGF-treated cells increases and maintains activation of EGFR, ERK1/2 and STAT3 but not Akt. In response to EGF, EGFR activation is unable to drive proliferation but activates cell scattering by inducing disruption of adherens junctions. In addition, EGF activates formation of lamelipodia structures and cell migration. Inhibition of EBP50 in non EGF-stimulated cells has the same
effects on cell scattering and migration as those observed with EGF alone. Cell dispersion due to EBP50 inhibition is abolished in the presence of gefitinib, an inhibitor of EGFR. Impact on cell scattering and migration is even greater when both EGF addition and EBP50 siRNA are combined. In vivo, delocalization of EBP50 normally expressed beneath the apical membrane is associated with EGFR expression in human biliary carcinoma tissues (p=0.002). Conclusion: These data provide evidence for a role of EBP50 in repressing EGFR-induced signaling pathway and migration. EBP50 at the membrane may be critical to confine cancer progression by preventing migration of biliary cancer cells.

559/B506
The Effects of Polyphenolic Induced Differentiation on HO-1, Human Melanoma Cells.
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Plant polyphenolic compounds have been found to exhibit anti-cancer effects such as changes of expressions in genes that control proliferation, differentiation and apoptosis. Consistent with our interest to investigate cellular differentiation as a therapeutic target for cancer, we investigated the effects of several synthetic polyphenolic compounds on HO-1 human melanoma cells. We now present data that shows VSP-15 (plant derived polyphenolic compound) exhibiting a dose- and time-dependent terminal differentiated effect on HO-1 cells. Proliferation assays and gene expression analysis using PCR arrays indicate the mechanism of action employed by the compound and indicate changes in gene expression indicative of the anti-proliferative and pro-apoptotic effects hypothesized. Morphological changes and melanin synthesis along with changes in the expression of the tumor suppressor genes MDA-7/IL-24 (melanoma differentiation gene-7/Interleukin-24) and p53 were also exhibited and were clear indication of VSP-15 induced differentiation.

560/B507
A Novel Role of RF-Cp145 in DNA Damage Induced Signaling.
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The Replication Factor-C (RF-C) is a heteropentameric protein that is relatively well conserved among eukaryotes. The major established role of RF-C complex is to load PCNA onto DNA. PCNA in turn functions as a sliding clamp that recruits DNA polymerases to allow DNA synthesis. We have discovered that the largest RF-C subunit, RF-Cp145, has a novel effect on DNA damage signaling. Chk1 and Chk2 kinases are activated by ATR/ATM kinases in response to DNA damage, and in turn propagate the downstream DNA damage response. We found that down-regulation of RF-Cp145 by siRNA in UV- but not in ionizing-irradiated cells prevents significatively Ser317 and Ser345 phosphorylation and activation of Chk1 and, in a less extend, prevents Thr68 phosphorylation of Chk2. The protein levels of other RF-C subunits are unaffected by RF-Cp145 siRNA. In contrast, RF-Cp145 protein levels are affected by the other RF-C subunits. Interestingly, the failure of Chk1 phosphorylation caused by RF-Cp145 down-regulation does not prevent UV induced phosphorylation of Ser15 in p53 nor does it prevent phosphorylation of H2AX which is increased when Chk1 is down-regulated. Furthermore, downregulation of ATRIP, a binding partner of ATR, destabilizes ATR and prevents UV induced phosphorylation of Chk1 and Ser15 phosphorylation of p53. It is therefore likely that RF-Cp145 does not act via ATR/ATRIP to inhibit selectively Chk1 phosphorylation, highlighting the emerging complexity of the DNA damage pathways. Finally, RF-Cp145 downregulation increases apoptosis, consistent with our published effect of RF-Cp145 on cell survival [1]. Eventually, RF-Cp145 plays a critical role in the UV surveillance during DNA replication by controlling the cell survival mainly through Chk1 phosphorylation. [1] Pennaneach V, Salles-Passador I, Munshi A, Brickner H et al. The large subunit of replication factor C promotes cell survival after DNA damage in an LxCxE motif- and Rb-dependent manner. Mol Cell 2001 Apr; 7(4):715-27.
Characterization of MUF1/LRRC41, a Binding Partner of Atypical Rho GTPases of the RhoBTB Family.

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Rho proteins have been implicated mainly in the regulation of the cytoskeleton, but also in vesicle trafficking, phagocytosis and transcriptional activation. RhoBTB proteins constitute a subfamily of atypical Rho GTPases represented by three isoforms in vertebrates. They consist of a GTPase domain followed by a proline-rich region and two BTB domains. BTB domains are involved in the formation of cullin 3-dependent ubiquitin ligase complexes therefore the function of RhoBTB proteins seems to be distinct from that of the classical Rho GTPases. In our working model RhoBTB proteins play roles in targeting of substrates for ubiquitinylation and degradation via cullin 3-dependent ubiquitin ligase complexes, but the substrates remain unknown. In order to identify binding partners (and possible substrates) of RhoBTB we performed a two hybrid screening on a mouse brain cDNA library and identified MUF1 (LRRC41) as a potential interaction partner of RhoBTB3. MUF1 is a leucine rich repeat containing protein that, interestingly, also contains a BC-box that serves as a linker in multicomponent, cullin 5-based ubiquitin ligases. We confirmed the interaction of MUF1 with all three RhoBTBs using immunoprecipitation. The interaction of MUF1 and RhoBTB3 seems to be direct, cullin 3 and 5 independent. We determined the expression profile of the LRRC41 gene using a Human Multiple Tissue Expression Array and RT-PCR and found that MUF1 is ubiquitously expressed, with the highest expression in testis. A GFP fusion of MUF1 localizes in the nucleus but RhoBTB3 causes it to be partially retained in the cytoplasm. MUF1 is able to make homodimers and appears to be degraded in the proteasome in a cullin 5 independent manner. This raises the question whether MUF1 and RhoBTB3 are together involved in multiprotein complexes containing cullin 3 and cullin 5 simultaneously. We envision two possible models: a) MUF1 is ubiquitylated by a cullin 3 complex dependent on RhoBTB, which acts as the substrate specific adaptor; b) RhoBTB is ubiquitylated by a cullin 5 complex dependent on MUF1, which acts as the substrate specific adaptor.

Para-Phenylenediamine-Induced Autophagy in Human Uroepithelial Cell Line Mediated Mutant p53 and Activation of ERK Signaling Pathway.

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Para-Phenylenediamine (p-PD) is a major aromatic amine that is widely used as a component of engineering polymers and commercial oxidative type hair dyes. Some epidemiologic studies suggested that the use of p-PD-based hair dyes might be related to increased risk of human malignant tumors including bladder cancer. However, it has been suggested that programmed cell death is composed of two different pathways for active cellular self-destruction as reflected by different morphology: apoptosis and autophagy. Previously, we have demonstrated that p-PD can induce apoptosis in urothelial cells. However, the molecular mechanism of p-PD on autophagy in human uroepithelial cells (SV-HUC-1) is still unclear. Our previously study had shown that the exposure of SV-HUC-1 cells to p-PD caused oxidative DNA damage and increased mutant p53 expression respectively, by using Comet assay and Western blotting. In this study, we demonstrate that p-PD could activate the extracellular signaling-regulated protein kinase 1/2 (ERK1/2) signaling pathway in p-PD-treated SV-HUC-1 cells. In addition, the substantial
morphological changes in cells after treatment of different concentrations of p-PD were examined. By electron microscopy, we found that the autophagosomes were increased in p-PD-treated SV-HUC-1 cells. Furthermore, our results showed incrementally increasing the p-PD concentrations, the expression of Beclin-1 and microtubule-associated protein light chain 3B (LC3B) which are important regulators of autophagosomes, were increased in a dose-dependent manner. In contrast, the inhibition of autophagy by treatment with MEK inhibitor (U0126), and the effect of p-PD on ERK1/2, Beclin-1 and LC3B proteins expression were suppressed, except mutant p53. Finally, our results provide the notion that p-PD can activation of ERK1/2 signaling pathway via mutant p53, led to stimulate autophagy in SV-HUC-1 cells. Therefore, these results provide us new insights for the understanding of the mechanism of p-PD-induced cell death in urothelial cells.

563/B510
Transforming Growth Factor β Inducible Early Response Gene 1(TIEG1) Is a Novel Substrate of Atypical Protein Kinase Cs (aPKCs).
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The protein kinase C (PKC) family of serine/threonine kinases consists of ten different isoforms that can be grouped into three classes denoted classical-, novel- and atypical PKCs. λι and ζPKCs constitute the atypical PKCs, which serve important roles during development and in processes subverted in cancer such as cell-and tissue polarity, cell proliferation, differentiation and apoptosis. In an effort to find novel interaction partners for aPKCs, a yeast two-hybrid screening of a HeLa cell cDNA library was carried out using the regulatory domain of λιPKC as bait. This screen yielded three independent clones of TIEG1 as a putative interaction partner for λιPKC. We have confirmed the interaction In Vitro and In Vivo using pull-down- and co-immunoprecipitation assays and confocal microscopy. In addition, we report that aPKCs phosphorylate the DNA binding domain (DBD) of TIEG1 on two critical residues. We further show that aPKC-mediated phosphorylation of TIEG1 affects its DNA binding activity, subnuclear localization and transactivation potential.

564/B511
Cinnamaldehyde Impairs High Glucose-Induced Hypertrophy in Renal Interstitial Fibroblasts.
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Cinnamaldehyde is a major and a bioactive compound isolated from the leaves of Cinnamomum osmophloeum kaneh. To explore whether cinnamaldehyde was linked to altered high glucose (HG)-mediated renal tubulointerstitial fibrosis in diabetic nephropathy, the molecular mechanisms of cinnamaldehyde responsible for inhibition of HG-induced hypertrophy in renal interstitial fibroblasts were examined. We found that cinnamaldehyde caused inhibition of HG-induced cellular mitogenesis rather than cell death by either necrosis or apoptosis. There were no changes in caspase 3 activity, cleaved poly(ADP-ribose) polymerase (PARP) protein expression, and mitochondrial cytochrome c release in HG or cinnamaldehyde treatments in these cells. HG-induced the extracellular signal-regulated kinase (ERK)/c-Jun N-terminal kinase (JNK)/p38 mitogen activated protein kinase (MAPK) (but not the Janus kinase 2/signal transducers and activators of transcription) activation were markedly blocked by cinnamaldehyde. The ability of cinnamaldehyde to inhibit HG-induced hypertrophy was verified by the observation that it significantly decreased cell size, cellular hypertrophy index, and protein levels of p27Kip1, p21Waf1/Cip1, collagen IV, and fibronectin. The results obtained in this study suggest that cinnamaldehyde in HG-induced hypertrophy in renal interstitial fibroblasts may serve a potential anti-fibrotic function through mechanism dependent of its inactivation of the ERK/JNK/p38 MAPK pathway.

565/B512
Flip S-Nitrosylation Modulates Protein Processing and NF-κB Activation through Changes with RIP1.
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Elevated nitric oxide (NO) production and FLICE-inhibitory protein (FLIP) expression have been associated with many human disorders such as cancer and cardiovascular diseases. However, their role and relationship in the disease process are not well understood. FLIP is a known regulator of death receptor (DR)-mediated apoptosis, but recent studies suggest that it also plays a role in inflammatory and proliferative pathways through NF-κB (Nuclear Factor kappaB) activation. DR signaling is tightly regulated since they are key players in determining cellular fate in response to cytokine stimulation. Disruption of these pathways is well documented in chemotherapy-resistant cancers and autoimmune diseases. The objective of this study was to determine the role NO has on FLIP signaling in the DR pathways. High levels of NO lead to an increase in protein S-nitrosylation, a post translational modification. Our studies show that the ability of FLIP to activate NF-κB is dependent upon FLIP S-nitrosylation. Mutation of cysteine residues at positions 254 and 259, prevent S-nitrosylation and abolishes its NF-κB inducing effect, as demonstrated by reporter gene assays. Immunoprecipitation, Western blot, and immunofluorescence studies further show that S-nitrosylation of FLIP modulates its interaction with a protein upstream of NF-κB activation, RIP1 (Receptor Interacting Protein 1). RIP1 also responds to DR-stimulation. We also show that the S-nitrosylation of FLIP is required for its processing to shorter forms suggesting that S-nitrosylation of FLIP promotes its ability to act as a substrate. Together, our results indicate a novel pathway for FLIP regulation of NF-κB through NO signaling. This provides a key mechanism for determining cell death and survival in the DR-mediated pathways.

566/B513
Loss of Calreticulin Suppresses NFκB Activity by Increasing IκB Stability.
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Calreticulin (CRT) is an endoplasmic reticulum chaperone which has high capacity for Ca2+ binding. Previously we showed that CRT knockout mouse embryonic fibroblasts (crt-/-) are resistant to UV-induced apoptosis. Recently, we also demonstrated that the activity of the PI3-kinase/Akt signaling pathway and the proteasome are significantly elevated in the CRT deficient cells. Given that NFκB signaling is induced by active Akt, enhanced ER stress and elevated proteasome activity, we hypothesized that NFκB signaling is activated in the absence of CRT. To test this hypothesis we compared the activity of a NFκB reporter gene in wild type (wt) and crt-/- cells. Surprisingly, we showed that the basal transcriptional activity of NFκB is significantly lower in the CRT deficient cells as compared to the wt cells. In addition, treatment of crt-/- cells with LPS failed to increase the transcriptional activity of NFκB to the level observed in the wt cells. Western blot analysis showed that the decreased NFκB activity was not due to a decrease in either the p65 or p50 subunits but was instead due to a significant increase at the level of IκBα protein level in the crt-/- cells. Interestingly, the level of IκBα and IκBβ mRNA were both significantly reduced in the CRT deficient cells. The observed increase in the IκB could be partly mediated by the rapid dephosphorylation and accumulation of IκB protein in these cells. Indeed, our data illustrated a significant increase in the activity of a serine-therionine phosphatase (PP2A). Furthermore, we showed that inhibition of PP2A by okadaic acid significantly reduced the IκBα protein level. In conclusion, we have demonstrated for the first time that loss of calreticulin diminishes NFκB activity by increasing IκBα stability due to activation of PP2A.

567/B514
Quantitation of ERK Activation via Automated Cellular Imaging and Analysis.
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Extracellular signal-regulated kinase (ERK) signaling controls cell growth, differentiation and survival. ERK is activated by dual phosphorylation on tyrosine and threonine residues (pERK). This pathway is often upregulated in tumors and is a target for cancer therapy. Evidence suggests that pERK may also represent a generic readout for GPCR activation. Our aim was to develop an immunofluorescence-based cellular imaging assay for ERK activation, in order to quantitate the activity of agents modulating pERK. We characterized multiple pERK antibodies via automated cellular imaging. By using image analysis algorithms, antibodies were eliminated if they displayed low signal, inappropriate localization, or minimal ERK activation upon treatment with ERK modulators. This approach enabled selection of an optimal pERK antibody for use in all further assay development. Treatment of HeLa cells with PMA (30min) in growth media (100nM max.) led to dose-dependent increases of nuclear and cytoplasmic pERK signal. Maximum increases in HeLa pERK nuclear and cytoplasmic intensity (compared to control) at 100nM PMA treatment were 115% and 87% respectively. A549, HeLa and HepG2 cells were used to generate ERK activation time courses for EGF and PDGF, showing maximal activation after 15-30min of treatment. Cell type-specific dose responses were also generated for PMA, EGF and PDGF. Dose-dependent inhibition of pERK was demonstrated using 1h pre-treatments (100µM max.) of LY294002, PD98059 and U0126, followed by 30min 100nM PMA activation. LY294002 produced minimal inhibitory effect in each cell type; IC50 values for PD98059 and U0126 ranged from 0.7-2µM and 67-740nM, respectively. We performed further experiments demonstrating the application of this assay to screen for GPCR activation in response to receptors activating three classes of G proteins: Gαs, Gαi and Gαq. In summary, we have developed of a robust assay for pERK screening via quantitative imaging. The assay utilizes sensitive, highly specific immunofluorescent detection of pERK, and may be used to rapidly visualize and quantify expression of this protein in a variety of cell types. This assay shows applicability for multiple ERK screening and drug discovery applications.

568/B515
The Atypical Mitogen-Activated Protein Kinase ERK3, a Novel Substrate for p-21-Activated Kinase Activity.
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Extracellular signal-regulated kinase 3 (Erk3/MAPK6) is a member of the mitogen-activated protein kinase (MAPK) family of serine/threonine kinases that transduce extracellular signals into discrete intracellular responses. The MAPK signal transduction pathways are among the most widespread and conserved mechanisms of eukaryotic cell regulation and their mechanism of action in cell cycle progression and differentiation are well established. The lesser known isoform, Erk3, differs from other MAPKs in that it does not contain a true canonical MAPK Th-X-Tyr motif, but contains a single phosphorylation site in its activation loop (Ser-Glu-Gly). Erk3 also has very restricted substrate specificity, and does not phosphorylate any of the known MAPK substrates, nor is it a target of the established MAPK kinases. Very little is known about the regulation, substrate specificity and physiological functions of Erk3. Recent studies suggest it may be involved in the regulation of the cell cycle and has been linked to some cancers. Our objective is to elucidate how Erk3 is regulated and the physiological implications of its regulation by identifying upstream effectors of Erk3. Using a high throughput protein array we found that Erk3 functions as a direct substrate for p-21 activated kinase 2 (Pak2), suggesting a relationship between Pak2 and Erk3. To confirm our findings, we purified full length Erk3 and performed phosphorylation studies confirming that Erk3 is indeed a direct In Vitro substrate for Pak2 kinase activity. In addition, we mapped the key residues phosphorylated by Pak2 using activation loop specific phospho-antibodies. An In Vivo confirmation of Pak2/Erk3 interaction was also demonstrated by immunoprecipitation assays. These finding support our hypothesis that Pak2 kinase activity may directly contribute to Erk3 activation. Furthermore, based on the similarity of Erk3 to Erk1/2, Pak2 might function analogously to MEK and be directly responsible for Erk3 activation. Further studies into how Erk3 is regulated will shed light into the role of the enzyme in cell cycle regulation and cancer.
Rewiring of JNK Map Kinase Signaling Pathway through Artificial Assembly of JIP-1 Scaffold Complex.
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Scaffold proteins are crucial for proper signaling of MAP kinase cascades in eukaryotic cells. Scaffold proteins are known to bind to component kinases of signaling pathway and to mediate signaling by formation of functional signaling complexes. It is also proposed that scaffolds could facilitate phosphorylation of member kinases, and presumably increase the efficiency and specificity of signaling. Although several scaffold proteins have been identified, it is still unclear how the scaffold proteins participate in the regulation of signaling and contribute to the signaling integrity. In this study, we have attempted to elucidate the assembly mechanism of mammalian scaffold protein, JIP-1 in JNK MAP kinase signaling pathway. First, JNK docking mutation was introduced in JIP-1 which disrupted signal transduction as well as interaction with JNK. Then, JNK MAP kinase and mutant JIP-1 were fused to nNOS and syntrophin PDZ domains, respectively, to reinforce JNK into JIP-1 complex via heterologous PDZ-PDZ interaction. We observed that the pathway output was functionally restored by rewiring JNK-JIP-1 docking using heterologous protein interaction, and that the non-natural assembly of scaffold complex mediated by heterologous PDZ domains is sufficient to restore JNK MAP kinase signaling. It seems that scaffold proteins serve as a platform for dynamic organization of signaling proteins and therefore, artificial assembly of scaffold complex should allow for the rewiring of signaling information.

The Tail Anchored Membrane Protein (SLMAP) Regulates Cell Growth by Targeting the Map Kinase Pathway and PP2A.
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The aim of this study is to evaluate the role of the tail anchored membrane protein (SLMAP) in cell signaling and growth. Knockdown of SLMAP using RNA interference was found to inhibit cell proliferation ~60% with no evidence of cell death and was associated with a ~68% inhibition of MEK activity and a marked hyperphosphorylation of p42/44. Although the MEK inhibitor (U0126) inhibited p42/44 activity, the phosphorylation of p42/44 remained higher in cells with reduced levels of SLMAP. Protein levels of p44 were increased ~85% while those of p42 were decreased ~64% upon SLMAP knockdown. The expression of the phosphatase MKP-1 that is believed to dephosphorylate p42/44 was unchanged while PP2A protein levels were decreased ~83% upon SLMAP knockdown. In contrast, overexpression of SLMAP depressed p42/44 phosphorylation and was associated with increased PP2A and p42 protein levels without any effects on MEK phosphorylation. Changes in SLMAP expression did not affect the mRNA levels of PP2A and p42. Confocal imaging of C2C12 cells demonstrated a colocalization of SLMAP, PP2A and striatin while immunoprecipitation assays revealed that SLMAP exists in complex with striatin and PP2A. These data demonstrate that SLMAP can critically impact cell growth through the MAP kinase pathway by specifically targeting MEK activity as well as PP2A and p42/44 protein levels. In conclusion, we propose a novel role for SLMAP as an adaptor/scaffold which serves to specifically integrate signaling through a multiprotein complex involving PP2A, striatin and components of the MAP Kinase pathway.

Role of Ppq1 Phosphatase in Mating Map Kinase Pathway in Budding Yeast.
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Cells process signaling information through biochemical networks of signal transduction proteins. Maintenance of signaling specificity by temporal and spatial regulation of protein complex formation is critical for cell survival. To understand regulation mechanism and kinetics of cell signaling, we have chose the mating MAP kinase signaling pathway in the budding yeast as a model. Upon stimulation by mating pheromone yeast cells transmit mating responses through the MAP kinase cascade composed of three kinases and Ste5 scaffold protein. Phosphatases are known to play a critical role for the precise regulation of signaling at each stage such as activation, duration, and adaptation. Identification and functional analysis of pathway-specific phosphatase is critical for understanding of the regulatory mechanism of cell signaling. We have developed a novel screening strategy for pathway-specific phosphatases, in which an entire repertoire of phosphatases in organism were tethered to the scaffold and the changes in the signaling response were monitored. Using the strategy, a putative Ser/Thr phosphatase, Ppq1, was identified as a negative regulator of the mating signaling. Results show that Ppq1 can directly interact with members of mating pathway. Catalytically active Ppq1 was able to lower the phosphorylation level of mating components In Vivo and in vitro, which suggests that Ppq1 might function as a negative regulator of mating MAPK pathway by dephosphorylating target protein. It seems that Ppq1 plays a critical role in the acute signaling propagation and signal adaptation via controlling the level of active MAP kinase.

572/B519
Systematic Quantification of Crosstalk and Feedback Interactions in Signal Transduction Networks.
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Historically, intracellular signal transduction has been characterized in terms of linear pathways, exemplified by the canonical mitogen-activated protein kinase (MAPK) cascades, such as the Ras → Raf → MEK → extracellular signal-regulated kinase (Erk) pathway in mammals. Our conceptual understanding of signal transduction networks now includes more complex interactions, including those between the classically defined pathways (crosstalk) and those responsible for feedback regulation/reinforcement; however, little has been done to move beyond hand-waving models of signaling networks to systematically quantify the relative magnitudes of classical, crosstalk, and feedback interactions. Through quantitative measurements and computational modeling, we recently characterized crosstalk mechanisms in the platelet-derived growth factor (PDGF) receptor signaling network, in which phosphoinositide 3-kinase (PI3K) and Ras/Erk pathways are prominently activated [Wang C-C, Cirit M, Haugh JM. PI3K-dependent crosstalk interactions converge with Ras as quantifiable inputs integrated by Erk. Mol Syst Biol, 5: 246 (2009)]. Unique in its coverage of time, dose, and molecular perturbation conditions, our data set was comprised of >3,000 biochemical measurements, yielding 169 processed data points that were used to constrain the accompanying model. We have since refined this approach with additional measurements that push even further the boundary of data-driven mechanistic modeling. With nearly double the number of data constraints, we have identified at least four distinct modes of negative feedback regulation and pinned down with even greater precision the magnitude of crosstalk from PI3K-dependent signaling to the Ras/Erk pathway. We further demonstrate that the model in its current state is a predictive tool that successfully forecasts outcomes of experiments that perturb the feedback structure of the network.

573/B520
PEA-15 Re-Programs the Output and Duration of Growth Factor Signaling.
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PEA-15 is a highly-conserved widely-expressed mammalian protein. Changes in PEA-15 expression are linked to insulin resistance, tumor cell invasion, and cellular senescence. The ERK1/2 MAP kinase pathway is a central regulator of cellular behaviors and PEA-15, an ERK1/2 binding protein, has profound effects on the activation and output of this pathway. Previous work has shown that increased PEA-15 expression leads to dramatically increased activation of the
ERK1/2 MAP kinase pathway. Here we have defined the mechanism whereby PEA-15 promotes and sustains growth factor signaling. In addition to increasing ERK1/2 activation, our data show that PEA-15 binds to ERK1/2 thereby preventing their membrane recruitment. In consequence, ERK1/2 are unable to threonine phosphorylate FRS2α, thereby prolonging its capacity to activate the MAP kinase pathway. We report that PEA-15 increases activation of the ERK1/2 MAP kinase pathway by interrupting a negative feedback loop formed by ERK1/2 phosphorylation of FRS2α. Furthermore, genetic deletion of FRS2α blocks the capacity of PEA-15 to activate the MAP kinase pathway. Thus, PEA-15 serves as a modulator of the classical MAP kinase pathway by preventing its localization to the plasma membrane thereby inhibiting, ERK1/2-dependent phosphorylation of FRS2α.

574/B521
Different Cell Proliferation Pathways Induced by FGF-2 through p27 Degradation via ERK1/2 and PI 3-Kinase in Human and Rabbit Corneal Endothelial Cells.
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The cyclin-dependent kinase inhibitor p27Kip1 (p27) is an important regulator of G1 progression. To induce cell proliferation, p27 must undergo proteolysis through its phosphorylation mechanism. Fibroblast growth factor 2 (FGF-2) is known to stimulate cell proliferation of rabbit corneal endothelial cells (rCECs) by degrading p27. We investigated the effect of FGF-2 on cell proliferation and p27 degradation in human corneal endothelial cells (hCECs) in both In Vitro and ex vivo studies. IL-1β induced FGF-2 expression through PI 3-kinase, as observed in rCECs. FGF-2, and IL-1β dramatically stimulated cell proliferation in hCECs; these effects were blocked by use of pathway specific inhibitors for PI 3-kinase and ERK1/2, respectively. We further defined whether the induction of cell proliferation by FGF-2 is caused by p27 degradation. Cells maintained in serum-starved medium were stained for p27 in the nuclei, but not for pp27Thr187. However, FGF-2 abolished the staining of p27, while nuclear staining of pp27Thr187 was observed. This effect of FGF-2 on p27 expression and on phosphorylation of p27 at Thr187 site was completely blocked by LY294002 treatment. We used immunoblotting to define whether both PI 3-kinase and ERK1/2 pathways are independently involved in cell proliferation induced by FGF-2, as observed in rCECs. In the FGF-2-treated cells, the activation of Akt, p38, and ERK1/2 were determined, and its activation was also completely blocked by LY294002: ERK1/2 activation involves the cell proliferation induced by FGF-2 as a downstream molecule to PI 3-kinase, which differs from rCECs in which the ERK1/2 pathway is parallel to PI 3-kinase pathway. With these results, we, thus, reveal for the first time that FGF-2 stimulates proliferation of hCECs through PI-3 kinase and its downstream target ERK 1/2 pathways and that FGF-2 significantly down-regulates p27 through the phosphorylation mechanism.

575/B522
Role of T34 Phosphorylation in Akt Dissociation from Membrane during Akt Activation.
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Akt is a critical serine-threonine kinase that controls cell survival, growth and differentiation. The regulation and function of Akt involves multi-step processes. Cytosolic Akt is recruited to the plasma membrane through the binding of the PH domain to membrane PIP3 generated by growth factor receptor stimulation. The membrane interaction causes interdomain conformational change of Akt allowing Akt activation by phosphorylation at T308 and S473 by PDK1 and mTOR. The activated Akt is then released from the membrane to phosphorylate downstream targets in various subcellular localizations. It has been demonstrated that the phosphorylation of T308 by PDK1 not only is important for Akt activity but also play a role in the detachment of Akt from the plasma membrane. However, detailed mechanism by which phosphorylated Akt dissociates from the membrane is still unclear. In the present study, we investigated the involvement of Akt phosphorylation in dissociation of Akt from the membrane. We found that Akt phosphorylation by PDK1 is required for Akt release from the membrane. We also found that PDK1 not only
phosphorylates T308 but also T34 in the PH domain. Biomolecular interaction analysis indicated that a mutant mimicking the phosphorylation of T34 (T34D) resulted in less membrane affinity compared to the wild type Akt. Consistently, the membrane translocation observed by time lapse fluorescence microscopy was impaired by the T34D mutation. Akt phosphorylation at both T308 and S473 in IGF-stimulated Neuro 2A cells was significantly inhibited in the T34D mutant, further supporting the impaired Akt interaction with membrane. The observed decrease in Akt-membrane interaction by T34 phosphorylation by PDK1 may be an important mechanism to mobilize activated Akt for downstream target protein phosphorylation inAkt signaling.

576/B523
Profiling of mTOR Phosphorylation Pathway in Rat Liver Using Mass Spectrometry.
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Cell growth is a very complex and highly coordinated process. The Mammalian Target of Rapamycin (mTOR) is at the center of a nutrient and growth factor-responsive cell growth signaling network. mTOR is the only known target of the naturally occurring fungicide, rapamycin. Previous studies have identified mTOR as a serine/threonine kinase that signals to a spectrum of downstream events, including activation of translation and ribosomal biogenesis, inhibition of autophagy, regulation of cell size and cell cycle progression. However, the functions of both proximal and distal components of the TOR pathway are incompletely understood and many potential substrates of mTOR and its downstream effectors remain unidentified. With recent advances in mass spectrometry technology, investigation of global and quantitative dynamics of phosphorylation events is possible. Our aim in this project is to gain a better understanding of how the mTOR pathway regulates cell growth by characterizing the global In Vivo phosphoproteome and its temporal dynamics under physiological conditions that modulate mTOR activity. Our work has thus far focused on developing a phosphoproteomic approach to enrich phosphorylated proteins from liver homogenates for identification of unknown phosphorylation sites regulated by mTOR signaling. By applying multiple proteomic chromatography methods and MS/MS sequencing, we have been able to identify known mTOR targets. We anticipate that our optimized phosphoproteomic strategy will allow us to enrich for and identify proteins from liver homogenates whose phosphorylation is modulated by the whole animal administration of rapamycin.

Cell Cycle Controls I (577 – 599)

577/B524
Caveolin-1 Binding with β-Dystroglycan Modulates Gαq-PLCβ1 Mediated Calcium Release in Smooth Muscle Cells.
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β-Dystroglycan (β-DG), the core transmembrane subunit of the dystrophin-glycoprotein complex (DGC), links the extracellular matrix to intracellular actin, and the DGC colocalizes with membrane caveolae arrays in smooth muscle. We tested whether association of caveolin-1 with the DGC dictates caveolae distribution, affecting pathways for [Ca2+]i mobilization by contractile agonists. Using confocal microscopy of cultured primary canine airway smooth muscle (ASM) and immunoblot analysis of canine and human ASM tissue, we observed colocalization of caveolin-1 and β-DG. Immunoprecipitation confirmed a direct association of caveolin-1 with β-DG. Disruption of filamentous actin with latrunculin (LAT) caused loss of organized caveolae membrane arrays.
(electron microscopy) and markedly reduced β-DG and caveolin-1 protein in isolated lipid raft-rich fractions. Actin-linked caveolae disruption also had significant functional consequence: in fura-2-loaded ASM cells, LAT decreased sensitivity to methacholine (MCh) (EC50Con = 50.3 ±14nM; EC50LAT = 220 ±24nM; p<0.01), and reduced maximum MCh-induced [Ca2+]i by 34% (p<0.01). To study the role of the interaction between β-DG and caveolin-1 we used lentiviral delivered shRNAi to stably silence β-DG in human ASM cells. In the absence of β-DG, caveolae distribution was clearly disrupted, and key GPCR machinery (PLCβ-1 and Gq) required for Ca2+ mobilization, was lost from caveolae microdomains as confirmed by subcellular fractionation. These changes correlated with a marked reduction in sensitivity to MCh-induced [Ca2+]i mobilization in β-DG null cells (EC50 = 50.3 ±14nM) compared to cells transduced with non-coding scrambled shRNAi (EC50 = 220 ±24nM; p<0.01). These data demonstrate for the first time a direct interaction between endogenous caveolin-1 and β-DG that is essential for caveolae-sequestration of signaling effectors for GPCR-mediated [Ca2+]i release, and indicate a critical role for the cortical actin cytoskeleton and the DGC in the spatial organization of caveolae that underpins [Ca2+]i release.

578/B525
Role of Intracellular S100B in the Transition of Myoblasts from Quiescence to Proliferation and from Proliferation to Quiescence, and in Apoptosis.
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S100B is a Ca2+-binding protein of the EF-hand type with both intracellular and extracellular regulatory roles. Within cells, S100B regulates the activities of certain enzymes and transcription factors, cell shape and migration, proliferation and differentiation. Recent evidence suggests that S100B is expressed in satellite cells (SCs), the quiescent stem cells located beneath the basal lamina surrounding each myofiber in skeletal muscles, as well as in myoblast cell lines, and that increases (by either stable or transient overexpression) or decreases (by RNA interference) in S100B levels result in the blockade and enhancement of myogenic differentiation, respectively. S100B stimulated the transcriptional activity of NF-κB by acting upstream of IKKβ thereby inhibiting the expression of the muscle-specific transcription factor, MyoD, and its downstream targets, myogenin and p21WAF1, and augmenting the expression of the anti-myogenic factor, Yin Yang1. Thus, S100B might exert a physiological role in the process of activation and expansion of SCs in the course of muscle regeneration, e.g. by reducing their premature differentiation. The ability of S100B to modulate MyoD expression raised the possibility that the protein might have a role in the transition of myoblasts from proliferation to quiescence and/or from quiescence to proliferation, as downregulation of MyoD expression is required for proliferating myoblasts to acquire a quiescent status, and activation of quiescent myoblasts results in expression of MyoD. We show here that increasing the S100B levels in myoblasts results in a reduced acquisition of quiescence (via ERK1/2-dependent activation of NF-κB) and an accelerated resumption of proliferation when the cells are being switched from quiescence medium to growth medium (via ERK1/2- and Akt-dependent activation of NF-κB). Moreover, enhancing S100B levels results in resistance to apoptotic stimuli, again in an NF-κB-dependent manner. Thus, intracellular S100B might have a role in the expansion of activated SCs during the muscle regeneration process.

579/B526
Phosphorylation of the Endoplasmic Reticulum Calcium Sensor STIM1 Underlies Suppression of Store-Operated Calcium Entry during Mitosis.
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When endoplasmic reticulum (ER) Ca2+ stores are depleted due to physiological Ca2+ release or pharmacological perturbation, Ca2+ influx via plasma membrane (PM) Ca2+ channels is activated by store-operated Ca2+ entry (SOCE). SOCE involves Orai1 Ca2+ influx channels and STIM1 ER Ca2+ sensors. ER Ca2+ depletion induces rearrangement of STIM1 from a diffuse
localization throughout the ER membrane into punctate structures near the PM, where it activates Orai1 channels. Thus, rearrangement of STIM1 into near-PM puncta is a critical step in the SOCE pathway. Interestingly, SOCE is strongly suppressed during mitosis, the only known physiological situation in which SOCE is negatively regulated; however, the mechanisms that underlie SOCE suppression during mitosis are unknown. We found that both endogenous STIM1 and expressed eYFP-tagged STIM1 (eYFP-STIM1) immunoprecipitated from mitotic but not interphase HeLa and HEK293 cells were recognized by the phosphospecific MPM-2 antibody, suggesting mitosis-specific phosphorylation of STIM1. We also found that rearrangement of eYFP-STIM1 into near-PM puncta in response to ER Ca2+ depletion was suppressed during mitosis. We therefore hypothesized that STIM1 phosphorylation underlies prevention of STIM1 puncta formation and suppression of SOCE during mitosis. MPM-2 recognizes phospho-serine or threonine followed by proline, and human STIM1 contains 10 occurrences of S/T-P, all downstream of amino acid 482. eYFP-STIM1 truncated at amino acid 482 (482STOP) was not recognized by MPM-2 when immunoprecipitated from mitotic cells, suggesting lack of phosphorylation. In support of our hypothesis, non-phosphorylatable 482STOP co-expressed with Orai1 rearranged into near-PM puncta in response to ER Ca2+ depletion in mitotic cells, and also significantly rescued mitotic SOCE. A combination of mass spectrometry and site-directed mutagenesis identified S486 and S668 as mitosis-specific phosphorylated residues, and mutation of both to alanine also resulted in partial but significant rescue of SOCE in mitotic cells. Therefore, our data suggest that phosphorylation of S486 and S668 underlies suppression of SOCE during mitosis, although additional phosphorylation sites are likely involved.

580/B527
Deletion Mutation Analysis Demonstrates a Biphasic Response to an Extracellular Calcium Pulse in Saccharomyces cerevisiae: Implications for a Mathematical Model of Calcium Homeostasis.

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We are interested in identifying and mathematically modeling the roles of the key proteins involved in regulation of calcium homeostasis in Saccharomyces cerevisiae over a time frame of thirteen minutes post calcium pulse. We assay this by transforming the yeast cells with a cytosolic aequorin reporter gene that gives off light in proportion to cytosolic calcium. Cells are placed in a luminometer and are pulsed with 100mM (final concentration) calcium chloride. Luminosity is followed over a 13 minute time period. Deletion strains of VCX1, PMR1, and PMC1 demonstrate an immediate phenotypic deviation from the wild type response to calcium, as they are not able to regulate their cytosolic calcium levels as well as the wild type strain (Marshall et al., 2009, Discrete and Dynamical Systems Series B, volume 12: 439). Wild type cells respond to this calcium pulse with a transient increase in their cytosolic calcium, and then the cytosolic calcium level of these cells returns to approximately pre-pulse baseline concentration, where it remains steady for the entirety of the assay time. At early time points after the calcium pulse, a calmodulin mutant (cmd1-6), deletions of CNB1, CRZ1, CMK1, and CMK2 behave as wild type, with cytosolic calcium rising to over 10 micromolar and then dropping back to approximately baseline. However, 6 minutes post calcium pulse, cytosolic calcium levels again rise above baseline in these mutants. Based upon this data, we propose that yeast respond immediately to intracellular calcium with sequestration into the endoplasmic reticulum/Golgi and vacuole via Pmr1p, Pmc1p, and Vcx1p, and that, at 6 minutes, a second response occurs mediated by calmodulin through calmodulin kinases, calcineurin, and Crz1p. We propose a new mathematical model to account for this biphasic response; the model incorporates a time delay in the feedback loop associated with the activity of calmodulin, calcineurin, and Crz1p.

581/B528
Clathrin Function Is Essential for Centrosome Maturation and Maintenance of Normal Centrosome Number.

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Clathrin (clathrin heavy chain 17 (CHC17)) is known for its role in endocytosis and membrane traffic. Recent studies further implicate CHC17 in centrosome maturation (Shimizu et al., J Cell Sci., 2009). Objective: The aim of this study is to demonstrate if and how clathrin affects mechanisms of centrosome duplication and maturation. Methods: HeLa cells stably expressing GFP-α-tubulin or centrin-2-GFP were depleted of clathrin proteins (CHC17, clathrin light chain (CLC), or muscle-enriched clathrin heavy chain (CHC22)) using siRNA-mediated RNAi and compared to cells treated with non-silencing siRNA. Cells were examined for the subcellular localization (immunofluorescence (IF) confocal microscopy) and centrosome protein levels (western blotting) for γ-tubulin, pericentrin and centrin-2. Results: Clathrin depletion in mitotic HeLa-GFP-α-tubulin cells resulted in an increase in amplified centrosomes (>2 centrosomes) as evidenced by γ-tubulin staining. Silencing of CHC17 or CLC alone resulted in a 2-fold increase in amplified centrosomes, and co-depletions (CHC17 + CLC) enhanced this effect by 3-fold. These results correlated with an increase in multipolar mitotic spindles. Furthermore, centrosome integrity was compromised as evidenced by a loss in the fluorescence intensity and focusing of γ-tubulin and pericentrin. In contrast, CHC22 depletion did not reveal significant spindle or centrosome defects. Interphase HeLa-centrin-2-GFP cells depleted of CHC17 revealed a 1.5-fold increase in centriole amplification (> 4 centrioles). Centrosome fragmentation was also evident by satellite formation of centrin-2-GFP foci and non-clustered pericentrin staining. Mitotic HeLa-centrin-2-GFP cells depleted of CHC17 and/or CLC also revealed amplified centrosomes and centrioles. Again, CHC22 depletion did not result in significant aberrations in centrosome or centriole number. Conclusions: CHC17 and CLC are revealed to be important in maintaining normal centrosome and centriole numbers and organelle maturation. This process may reflect the interaction between clathrin and cyclin G-associated kinase (GAK) as reported by Shimizu and colleagues (2009).

582/B529
Spatial Regulation of the Spindle Assembly Checkpoint in Aspergillus and Disruption of BubR1 Localization by a Gamma-Tubulin Mutation.
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We have previously found that mutations in the Aspergillus nidulans γ-tubulin gene, mipA, cause defects in mitotic regulation. To clarify the role of γ-tubulin in mitotic regulation, we have C-terminally tagged a number of mitotic regulatory proteins under the control of their own promoters and examined, by time-lapse microscopy, their localization patterns in control strains and in strains carrying a γ-tubulin mutation, mipAD159. Here, we report the localization patterns of the spindle assembly checkpoint (SAC) proteins Mps1, Mad2, Bub3, and BubR1. In interphase, as recently reported (De Souza et al., 2009, Mol. Biol. Cell, 20:2146-2159) Mad2 is located in dots at the nuclear periphery. Mps1 localizes to the spindle pole body/kinetochore (SPB/K) complex beginning in S/G2, with the fluorescence gradually becoming brighter as the cell proceeds through G2. Bub3 is present in the nucleoplasm in interphase, and BubR1 does not show a distinctive localization. It is excluded from nuclei and appears to be diffuse in the cytoplasm. at mitotic onset, Mad2 and BubR1 translocate to the SPB/K complex and Bub3 is reduced in the nucleus except at the SPB/K complex. Thus, all four components come together at the SPB/K complex at mitotic onset, allowing formation of the mitotic checkpoint complex (MCC). Since the MCC components are physically separated until mitotic onset, it follows that the SAC cannot be activated until this point and, thus, that there is a precise spatial regulation of the SAC. In a mipAD159 strain, Mps1 and Bub3 localize normally, but BubR1 fails to localize to the SPB/K complex in a subset of nuclei. This indicates an important role of γ-tubulin in the accumulation of BubR1 upon mitotic onset and shows that BubR1 is not required for localization of Mps1 or Bub3 to the SPB/K complex. Previous work in our lab shows that mipAD159 causes constitutive activation of the anaphase promoting complex/cyclosome (APC/C) in a subset of nuclei. Since BubR1 has two KEN boxes that are predicted to be recognized by the APC/C, it is probably being continuously
targeted for degradation by the APC/C in these nuclei. Supported by grant GM031837 from the NIH.

583/B530
Testing a Licencing Model for DNA-Damage Induced Centrosome Amplification.
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The centrosome, which is the major microtubule organizing center in eukaryotic cells, duplicates once, and only once, during each cell division cycle. This limitation is due to a centrosome-autonomous inhibition of reduplication after S phase. However, centrosome overduplication can occur in cells exposed to various forms of DNA damage. In these cells, centrosome duplication is uncoupled from the DNA replication cycle and this can result in the formation of multipolar mitotic cells, and in some cases cell death. To understand how DNA damage-induced centrosome amplification occurs, we have explored two hypotheses. According to an ‘activation model’ an activating signal stimulates centriole duplication, whereas in a ‘licencing model’, an inhibitory signal that normally limits the duplication of the centrosome to once per cell cycle is lost as a result of DNA damage. In this study we investigated which model applies to DNA damage-induced centrosome amplification. Synchronized populations of human U2OS osteosarcoma cells were irradiated at defined stages in the cell cycle and then fused using polyethylene glycol treatment. Live cell imaging of fusions between wild-type U2OS cells and Nedd1-GFP or Centrin2-GFP expressing U2OS cells revealed that the Nedd1-GFP or Centrin2-GFP incorporates into the centrosomes of the wild-type fusion partner. These findings were consistent with our preliminary fluorescence recovery after photobleaching (FRAP) analysis of Nedd1 and Centrin2, which suggests that both proteins recover rapidly. Therefore, we used immunofluorescence microscopy to score the number of heterotypic cell fusions with amplified centrosomes. We saw significantly more centrosome amplification in cell fusions of irradiated G2 cells than in irradiated G2-unirradiated G2 fusions. These findings suggest that an inhibitory signal intrinsic to G2 centrosomes is lost upon IR treatment and that this leads to the overduplication of only the irradiated G2 centrosomes in fused cells. We suggest that a licensing model applies to centrosome amplification after DNA damage.

584/B531
Causes and Consequences of Centrosome Amplification.
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Genome instability and aneuploidy are common characteristics of many tumours. Supernumerary centrosomes are also observed in many tumour cell lines, leading to the model that during mitosis, cells with multiple centrosomes fail to divide properly. This may result in progeny with an unbalanced complement of chromosomes. One model for how centrosome amplification occurs is that centrosomes amplify during an extended cell cycle arrest following DNA damage. We wished to investigate mechanisms by which centrosome/centriole amplification can occur and the outcome of such amplification. A range of candidate centrosomal genes were also screened by quantitative RT-PCR to investigate possible differences in transcriptional levels after DNA damage. The mRNA levels of several candidates altered following ionising radiation. We will examine how these candidates affect centrosome activity after ionising radiation. Inhibition of CDK1 leads to an extended delay in G2 phase, as cells cannot enter mitosis. Following CDK1 inhibition by the small molecule RO3306 in untransformed hTERT-RPE1 cells, we observed up to 26% of cells with more than two centrosomes after 30 hours. After RO3306 washout, ~20% of cells quickly re-entered the cell cycle into mitosis, with many of the cells with multiple centrosomes forming multipolar spindles when released from the cell cycle block. Immunoblot analysis of phospho-H2AX levels following RO3306 treatment indicated that DNA damage had not occurred. In addition, we have generated an inducible PLK4 allele in human osteosarcoma U2OS cells. PLK4 overexpression induces rosettes of daughter centrioles around a single mother centriole. These centriole rosettes are able to form a bipolar spindle leading to a seemingly
normal cell division. However, the subsequent cell division is not so organised. Multipolar spindles form, or bipolar divisions with multiple centrosomes occur leading to a disorganised cell division. Using live cell imaging, we aim to follow the fate of cells in which we have caused multipolar spindles and determine if and how they can complete mitosis. We also aim to determine what death mechanisms are involved in the cells which fail to complete mitosis.

585/B532
Cdk2 and Cdk4 Prevent Centrosome Amplification by Restricting Excessive Centrosome Cycle Licensing and Duplication.
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The centrosome maintains euploidy by directing the formation of two spindle poles and equal segregation of sister chromatids during mitosis. To maintain a normal content of one or two centrosomes, the centrosome duplication cycle is tightly regulated. Aberrant centrosome duplication or cytokinesis results in centrosome amplification (CA) and aneuploidy, a major form of chromosome instability (CIN). As most human cancers display elevated frequencies of CA and CIN, they may contribute to the genesis and sustenance of most cancers. Cyclin E1-//-E2-//- or Cdk2-//- mouse embryonic fibroblasts (MEFs) grow slowly and harbor normal centrosomes, suggesting redundancy in the regulation of the cell and centrosome duplication cycles. Here we find that ablated Cdk2 or Cdk4 abrogates centrosome amplification and chromosome instability in p53-/- MEFs. They both inhibit centrosome amplification by restricting centriole reduplication in p53-/- MEFs. Ablated Cdk2 or Cdk4 regulate normal licensing by restoring normal levels of NPMT199 in p53-/- MEFs to wild-type levels. Our findings demonstrate that Cdk2 and Cdk4 restrict centrosome amplification.

586/B533
A Novel Microtubule-Binding Agent Induces Centrosome Hyperamplification and Multipolar Mitosis Causing Mitotic Death, Slippage and Aneuploidy to Facilitate Apoptosis.
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Aberrant spindle organization leading to chronic mitotic arrest and eventually, apoptosis is a characteristic feature of most tubulin-binding drugs. However, the exact nature and mechanism of the arrest, and factors dictating abnormal spindle construction remain elusive. Precise regulation of the centrosome duplication cycle is a prerequisite for the prevention of chromosome missegregation that results in aneuploidy. Although induction of centrosome amplification and aneuploidy have been long considered as characteristics of malignant tumors, it is becoming appreciated that induction of high-grade aneuploidy is in fact, death-inducing. Here we show that a novel microtubule-modulating agent, 9-bromonoscapine, targets the centrosome duplication cycle, uncoupling it from DNA replication during early S phase resulting in centrosome hyperamplification and formation of aberrant multipolar spindles. Using synchronized HeLa cells, cell-cycle phase-specific treatments show that excluding S and early G2 phase from drug treatment results in loss of both spindle multipolarity and consequent cell death. These data demonstrate that the drug cytotoxicity window is the S/early G2 phase during which 9-bromonoscapine alters the expression of an array of key centrosome duplication regulators, such as Plk4, cdk2/cyclin A/E, pRb, and Aurora A. Supernumerary centrosomes and multipolarity lead to an activated mitotic checkpoint resulting in a prolonged mitotic arrest. The durability of this mitotic arrest appears to be a determinant of drug-induced cell death. Complex signaling networks then dictate decisions made by cells to choose among mitotic death, mitotic exit, abnormal cytokinesis and ensuing aneuploidy, as various available strategies to die. Our data show that drug-induced centrosome hyperamplification, causing multipolar mitosis and high-grade aneuploidy followed by cell death, is a novel chemotherapeutic strategy.

587/B534
cIAP1 Is a New Activator of E2F-1 Transcription Factor.
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The inhibitor of apoptosis protein cIAP1 (cellular inhibitor of apoptosis protein-1) is an E3 ubiquitin ligase that displays oncogenic properties. In the cytoplasm, the protein is a potent regulator of TNF receptor family and NF-κB signalling pathways. In many cell types, however, cIAP1 is mainly expressed in the nucleus which is not in accordance with its cell signalling activity. The study objective was to investigate the nuclear function of cIAP1. We showed that cIAP1 is a regulator of cell proliferation. We demonstrate by GST-pull down and immunoprecipitation of endogeneous proteins that cIAP1 directly interacts with E2F1 transcription factor. It stimulates its transcriptional activity in a gene reporter assay, as efficiently as the well-known E2F-1 co-activator DP1. We demonstrate by ChIP experiments that cIAP1 is recruited, along with E2F1, on E2F binding site of the promoter of cyclin E and A, in a cell cycle dependent manner. cIAP1-siRNA inhibits the recruitment of E2F-1 on cyclin promoters and reciprocally, E2F-1 si-RNA blocks the DNA recruitment of cIAP1. cIAP1 can stimulate the cyclin E promoter in gene reporter assay which is completely abolish by a mutation into E2F binding site. Lastly, we demonstrated that cIAP1 modulates the transcriptional expression of cyclin E and interferes with the progression of cell into S phase of cell cycle. The ability of cIAP1 to stimulate E2F-1 transcription activity may account for its oncogenic properties.

588/B535
CKβ8/CCL23 Induces Up-Regulation of Cyclins via Gi/Go Protein/PLC/PKCδ/ERK Leading to Cell Cycle Progression.
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CKβ8/CCL23 is a CC chemokine and alternative splicing of the CKβ8 gene produces two mRNAs that encode CKβ8 and its isoform CKβ8-1. Chemokines play a critical role in leukocyte trafficking and development of inflammation and a kind of chemokines are also known to be involved in cell proliferation. To investigate implication of CKβ8 and CKβ8-1 in cell proliferation, we examined the effects of CKβ8 and CKβ8-1 in cell cycle. CKβ8 and CKβ8-1 induced cell cycle progression. Next, we investigated whether MAPKs are involved in CKβ8- and CKβ8-1-induced cell proliferation. CKβ8- and CKβ8-1-stimulated cells showed phosphorylation of ERK1/2 and inhibitor study indicated that CKβ8- and CKβ8-1-induced activation of ERK1/2 is mediated by Gi/Go protein, PLC and PKCδ. CKβ8 and CKβ8-1 regulated expression of cell cycle regulators including cyclin D3 and cyclin B1 and immediate early response genes such as c-Myc and Egr-1. These results suggest that CKβ8 and CKβ8-1 are involved in cell proliferation by modulating cell cycle regulators.

589/B536
The Abbreviated Self-Renewal Cell Cycle of Human Embryonic Stem (hES) Cells Depends on Cyclin D2 Related Activation of the CDK Substrate p220/NPAT at Histone Locus Bodies.
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We have examined fundamental parameters of hES self renewal, and the molecular mechanisms that control progression into S phase. BrdU incorporation, FACS analysis, and Ki67 staining revealed that the G1 phase of hES cells is abbreviated (~2.5 h). Autocrine mechanisms sustain this abbreviated cell cycle and mitotically synchronized hES cells are competent to initiate two consecutive S phases in the absence of exogenous growth factors. Differentiation of hES cells alters cell cycle kinetics by lengthening G1 within 72 h. Thus, the abbreviated G1 phase is
functionally linked to pluripotency. We also find that histone H4 gene expression is cell cycle regulated in hES cells and linked to CDK phosphorylation of the NPAT/HiNF-P complex interacting with histone genes at Histone Locus Bodies (HLBs). We show that HLBs in hES cells represent cell cycle dependent microenvironments that integrate transcriptional initiation and 3' end processing of histone gene transcripts. Molecular analyses demonstrate that Cyclin D2 is prominently expressed in pluripotent hES cells, but cyclin D1 eclipses cyclin D2 during differentiation. Depletion of cyclin D2 or NPAT causes a cell cycle defect in G1 and diminishes (i) phosphorylation of NPAT, (ii) histone H4 expression and (iii) S phase progression. Thus, cyclin D2 and NPAT are principal cell cycle regulators that determine competency for self-renewal in pluripotent hES cells. While pRB/E2F checkpoint control is relinquished in human ES cells, fidelity of physiological regulation is secured by cyclin D2 dependent activation of the NPAT/HiNF-P mechanism that may explain perpetual proliferation of hES cells without transformation or tumorigenesis.

590/B537
Transcription Factor II(TFII) Expression of Liver Regeneration and Development in Mouse.
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BACKGROUND and PURPOSE: Liver has a strong ability of regeneration after hepatic injury or resection. It has been reported that growth factors such as hepatocyte growth factor(HGF), epidermal growth factor(EGF), transforming growth factor(TGF) and cytokines including interleukins -1(IL-1) and -6(IL-6), tumor necrosis factor-alpha(TNF-α) participate in liver regeneration. However, the correct mechanism of liver regeneration is not fully understood. To understand liver regeneration process, we compare the genes involved in liver regeneration and development. METHOD: We used ICR mice and divided into 4 groups; fetus, adult mice operated hepatectomy, adult mice treated carbon tetrachloride (CCl4), and normal adult mice. Developing livers are obtained from E10.5, E12.5, and E14.5. Regenerating livers are acquired from operated adult mice and CCl4 treated mice. Hepatectomy is 68% resection of adult liver and liver cirrhosis is induced by intraperitoneal (IP) injection of CCl4. After hepatectomy and carbon tetrachloride treatment, TFII protein expression of 4 groups are analyzed by western blot. RESULT: TFII expression of regenerating liver reveals that genes associated with metabolism are somewhat down-regulated. The genes related to DNA synthesis and cell cycle regulation are up-regulated. We studied the expression TFII protein of developing liver, the data suggest that some genes associated with regeneration are also expressed in the mouse fetus liver. Our study suggests that these genes may have crucial roles in the liver growth.

591/B538
The Mitosis to Interphase Transition Is Coordinated by Crosstalk between the SIN and MOR NDR Kinase Pathways in the Fission Yeast S. Pombe.
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The NDR/LATS family of kinases are highly conserved from yeasts to humans and function at the end of related signaling cascades. Fission yeast, like humans, possess two NDR kinase pathways, one called the SIN (LATS pathway in humans) is required for cytokinesis and the other called the MOR (human NDR1/2 pathway) is required for interphase polarity. In animal cells the LATS and NDR1/2 pathways also function in cytokinesis and cell polarity respectively as in yeast. In addition both these pathways have been implicated in regulation of tumor formation. However it is unclear whether the activities of these pathways are coordinated. Our work in fission yeast shows that cross-talk between the SIN and MOR pathways is required to coordinate the discrete programs for cytoskeletal remodeling that exist in interphase and mitosis. at the end of mitosis the SIN pathway not only promotes cytokinesis, but also inhibits the MOR pathway to block reformation of the interphase cytoskeleton until cytokinesis is complete, presumably to keep the
MOR from competing for shared components such as actin. SIN signaling inhibits the kinase activity of the most downstream MOR component, the Ndr-family kinase Orb6. We show that the SIN regulates the localization of the upstream activators of Orb6 called Nak1 and Pmo25, providing a potential mechanism by which the SIN inhibits Orb6 activity. Inactivation of the MOR pathway stabilized the actomyosin ring in SIN mutants allowing them to complete cytokinesis. Together our results identify a mutually antagonistic relationship between the SIN and MOR pathways that is crucial for establishing and maintaining the discrete cytoskeletal arrangements that occur in cytokinesis and interphase. Given the functional conservation of these pathways our results may define general mechanisms by which mitotic and interphase cytoskeletal arrangements are coordinated.

592/B539
The Spindle Position Checkpoint Requires Positional Feedback from Cytoplasmic Microtubules.
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The objective of mitosis is to provide a copy of the genome to each progeny of a cell division, which requires the separation of duplicate chromatids by the spindle apparatus, and the delivery of one set of chromosomes to each of the daughter cells. In budding yeast, the fidelity of chromosome delivery depends on the spindle position checkpoint, which inhibits mitotic exit until one end of the anaphase spindle arrives in the bud. Here, we tested the hypothesis that activating and maintaining the spindle position checkpoint depends on persistent interactions between cytoplasmic microtubules and the mother-bud neck, the future site of cytokinesis. We used laser ablation to disrupt microtubule interactions with the bud neck, and we found that loss of microtubules from the neck leads to mitotic exit in checkpoint-activated cells. Our findings suggest that cytoplasmic microtubules are used to monitor the location of the spindle in the dividing cell, and in the event of positioning errors, relay a signal to delay the completion of mitosis until the spindle is appropriately positioned.

593/B540
Regulation of G1-S Transition by a Hyperfused Form of Mitochondria.
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Mitochondria undergo fission-fusion events that render these organelles highly dynamic in cells. By using tissue culture cells and drosophila as model systems we report a relationship between mitochondrial form and cell cycle control at the G1-S boundary. Live cell imaging of mitochondria in tissue culture cells revealed that at G1-S transition mitochondria convert from isolated, fragmented elements into a hyperfused, giant network. The network was electrically continuous and exhibited greater ATP output than mitochondria at any other cell cycle stage. Depolarizing mitochondria at early G1 specifically prevented progression into S, suggesting that increased mitochondrial function at G1-S is necessary for progression into S phase. Inducing hyperfusion of mitochondria in quiescent cells (i.e., maintained without growth factors) by acute inhibition of dynamin-related protein-1 (DRP1) led the cells to begin replicating their DNA, and coincided with buildup of cyclin E, the cyclin responsible for G1 to S phase progression. Prolonged or untimely formation of hyperfused mitochondria in cells, through chronic inhibition of DRP1, caused defects in mitotic chromosome alignment and S phase entry defect characteristic of cyclin E overexpression. As these defects were found to be dependent on p53 in tissue culture cells we hypothesized that in absence of p53 hyperfusion of mitochondria would induce hyperproliferation by overexpressing cyclin E. Indeed, constitutive maintenance of hyperfused mitochondria in drosophila, by inducing DRP1 knockout, leads to aberrant increase in cyclin E levels and activity in the somatic follicle in the ovary. This results in hyper-proliferation of the DRP1 knockout somatic follicle cells and they escape differentiation into epithelial cells, thus, producing aberrations in ovariole formation. Based on these findings, we conclude that changes in mitochondrial morphology during the cell cycle underlie proper cell cycle progression. Moreover,
we propose that a hyper-fused mitochondrial system with specialized properties is formed at G1-S and is linked to cyclin E buildup for regulation of G1 to S progression.

594/B541  
**Cyclin Limitation and Nuclear Size Promote Asynchronous Division of Nuclei in a Common Cytoplasm.**  
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Genetically identical cells that share the same environment vary in the length of their cell division cycles. In some multinucleate cells, variable and asynchronous nuclear division cycles are observed in nuclei residing in the same cytosol. How temporal variation is generated and tolerated within the precise regulatory controls of the cell cycle is not well understood. Here we show in a model multinucleate fungus, *Ashbya gossypii*, that variability and asynchrony in nuclear division timing require an Rb-analogue, Whi5p, a transcriptional repressor of the G1/S transition. Mutants lacking Whi5p or proteins that act upstream or downstream of Whi5p all have significantly more synchronous nuclear division cycles. Unlike in *Saccharomyces cerevisiae*, in *A. gossypii* Whi5p is nuclear across all stages of the cell cycle, indicating that Whi5p is inhibited within the nucleus. Doubling the gene dose of CLN1/2 is sufficient to induce partial synchrony, suggesting that Whi5p may contribute to variable cycle times by limiting the levels of Cln1/2p. Additionally, we observed that nuclear volume is highly variable in a population of nuclei and not equally partitioned in mitosis. We use mathematical modeling to examine how limited G1 cyclin levels can combine with variable nuclear size to promote heterogeneous nuclear division kinetics. This work indicates that nuclear geometry can amplify cell to cell variability and influence timing of the division cycle.

595/B542  
**Nup2, and a Novel Nucleoporin NupA, Have Essential Functions at Mitotic Chromatin Controlled by the Nima Kinase.**  
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In *Aspergillus nidulans*, the NIMA kinase is required for the transition from G2 into mitosis by regulating the reversible mitotic disassembly of nuclear pore complexes (NPCs), the gateways between the nucleus and the cytoplasm. How NIMA regulates this process, and the mitotic functions of nuclear pore proteins, are not understood. The potential mitotic role of Nup2 is of particular interest because it has the unique ability to transfer from NPCs exclusively to chromatin at mitosis, a translocation observed in *A. nidulans* and vertebrate cells. This remarkable feature, and the fact that Nup2 is essential in *A. nidulans*, suggest Nup2 might play essential mitotic roles downstream of NIMA that are conserved from fungi to humans. We have determined that induction of NIMA promotes both Nup2 phosphorylation and its relocation from NPCs onto chromatin, even in S-phase arrested cells. To help define Nup2’s function at mitotic chromatin, we affinity purified Nup2 from G2 and mitotic samples. Mass spectroscopy identified a novel nucleoporin, NupA, that copurified with Nup2 from both samples. Like Nup2, NupA transfers from NPCs in interphase to mitotic chromatin and is also essential. Deletion analysis and heterokaryon rescue revealed that the function of NupA is to tether Nup2 to both the NPC and mitotic chromatin. Like Nup2, NupA is highly phosphorylated during mitosis and deletion of either does not cause defects in nuclear transport but does cause identical mitotic DNA segregation defects. These studies demonstrate that the mitotic promoting functions of NIMA involve not only disassembly of NPCs but also the translocation of Nup2, and the newly discovered NupA protein, onto mitotic chromatin. The studies also reveal that the function of NupA is to locate Nup2 to NPCs and mitotic chromatin where both are required for normal mitosis.

596/B543  
**Evolution of Combinatorial Phosphoregulation in the Cell Cycle.**
Complex cellular processes are often orchestrated by master regulatory kinases. As organisms evolve, these kinases coordinate the functions of a shifting set of substrates, and as new kinases emerge through gene duplication and divergence, combinatorial control of substrate activities can provide a selective advantage. To better understand the evolution of phosphoregulation, we analysed the position and conservation of large numbers of phosphorylation sites for the cyclin-dependent kinase, Cdk1, and the related kinase, Ime2, in the budding yeast Saccharomyces cerevisiae. We used quantitative mass spectrometry to identify about 300 Cdk1 substrates and 400 Ime2 substrates in vivo. We found that Cdk1 and Ime2 phosphorylate a highly overlapping set of proteins at distinct sites that often cluster together. Comparisons of S. cerevisiae kinase substrates with orthologues throughout the ascomycete lineage allowed inference of the evolutionary dynamics of phosphoregulation. The precise position of most phosphosites is not conserved; instead, they often drift within rapidly evolving disordered regions. We propose that most phosphoregulation depends on simple non-specific mechanisms involving the disruption of protein complexes or the generation of interactions with modular phosphopeptide-binding domains. The gain or loss of phosphosites in rapidly evolving regions could facilitate the evolution of kinase signaling circuits and enable the integration of inputs from multiple kinases as new circuits evolve.

597/B544

Vasa Is Essential for Cell Cycle Progression.
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Several genes expressed in germ cells and stem cells are conserved throughout the animal kingdom. Recently some of these genes were found to function more broadly than originally anticipated. Vasa is a conserved ATP-dependent RNA helicase that functions in germ cells and many stem cells from cnidarian to mammals. Although implicated in translational regulation, the function of Vasa is poorly understood. Here we report a novel function of Vasa in sea urchin embryos. We found that Vasa protein is present in all blastomeres of the early embryo, and that its abundance oscillates with the cell cycle. During M-phase, Vasa selectively accumulates in the perinuclear region, and then enters the nuclear region following nuclear envelope breakdown. It associates with the spindle and the separating sister chromatids at metaphase, and then quickly disappears after telophase. Vasa organization during M-phase appears to be independent of microfilaments, but requires microtubules. Inhibition of Vasa protein synthesis slowed the cell cycle, maybe caused by chromosomal segregation deficiency. Further, the knock down of Vasa protein reduces general translation. These results demonstrate an active and selective role for an RNA-helicase in microtubule dynamics that implicates a regulatory role for RNA structure in spindle function. In addition, this work describes a novel function of Vasa as a general translation factor in embryonic stem cells.

598/B545

CyclinB1 Is Essential for the First Divisions of the Mouse Embryo.
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The Cyclin B-Cdk1 complex is required in all eukaryotes to initiate entry into mitosis. Although it has been shown that mice lacking Cyclin B1 cannot be detected in late embryogenesis, the stage in development at which Cyclin B1 becomes essential is unknown. Furthermore, many functional aspects of Cyclin B1, including its highly dynamic subcellular localization, are not yet fully understood. Most of the data on Cyclin B1 function and localization have been generated in cultured cell lines in which the effects of Cyclin B1 depletion by RNAi are difficult to interpret due to variable levels of knock down. To set up a system with a clean Cyclin B1 null background we have re-established a knock-out mouse line that was generated by Michael Brandeis and
colleagues in 1998 (a kind gift of Dr Tim Hunt). We have set up live-imaging of pre-implantation embryos to analyse the phenotype of Cyclin B1 null cells in greater detail, as well as to be able to use such cells to study Cyclin B1 function by re-expressing mutants of Cyclin B1. Here we show that Cyclin B1 null embryos arrest at day 2 of development after only one or two divisions. Thus Cyclin B1 is the earliest Cyclin required for development to date. Using live imaging we characterise the arrest phenotype and show at which stage in the cell cycle the arrest occurs.

599/B546
Role of MAPKAP-K2 in the G2/M Transition and Apoptosis in Response to Oxidative Stress.
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The p38 MAPK is involved in the transmission, amplification and diversification of extracellular signals by initiating different cellular responses including the regulation of cell proliferation. p38 is phosphorylated and activated in response to cellular stress by MKK3/6 and in turn phosphorylates a number of substrates, including MAPKAP-K2 (MK2). It has been demonstrated that MK2 can directly phosphorylate Cdc25B and Cdc25C, inhibiting the G2/M transition. In addition, a similar mechanism of cell cycle control has been described for this kinase in fission yeast: Srk1, a downstream kinase of yeast Sty1 (p38 MAPK), controls mitotic entry by directly phosphorylating and inhibiting Cdc25 during osmotic stress. Here we show that MK2 and MK3 are necessary to inhibit the G2/M transition in response to non-genotoxic stress. Moreover, stress conditions induce MK2-dependent apoptosis through activation of the E2F1 transcription factor.

Meiosis and Mitosis I (600 – 627)

600/B547
DALEK6: The Missing LINC for Mammalian Meiosis.
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A striking characteristic of meiotic prophase is an association between the ends of condensed chromosomes with the nuclear envelope (NE). During the zygotene stage of prophase these NE-attached telomeres are transiently redistributed towards the centrosomal pole of the nucleus, forming a structure called the meiotic bouquet that is believed to facilitate homologous chromosome alignment. In mammals the inner nuclear membrane protein Sun1 is required for this chromosomal-NE attachment. Sun1 is a constituent of the LINC-complex that together with outer nuclear membrane (ONM) KASH proteins forms a translumenal link spanning the NE. The LINC-complex physically connects the cytoskeleton to the nucleoskeleton and, in the case of meiosis, to the chromosomes. What remains unclear is the identity of ONM proteins required for bouquet formation and how they associate with the cytoskeleton to elicit chromosome movement. We have identified a novel ONM protein whose lumenal domain interacts with Sun1 and cytoplasmic domain binds the dynein motor complex. We have named this protein DALEK6. Expression of DALEK6 appears restricted to the testis in males, where it is found to colocalize with Sun1 at telomeres in primary spermatocytes. It is our hypothesis that DALEK6 is the mammalian constituent of the LINC complex that utilizes its association with the minus-end motor, dynein, to generate the meiotic bouquet. We are now in the process of deriving knockout mice in order to examine the role of DALEK6 in vivo.

601/B548
Mechanisms of Chromosome Segregation on Acentrosomal Spindles during C. elegans Oocyte Meiosis.
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Although centrosomes serve to organize microtubules in most cell types, oocyte spindles form in their absence. We are investigating how these acentrosomal spindles assemble and...
promote proper chromosome partitioning, using *C. elegans* oocyte meiosis as model. 

Recently, we reported that oocyte spindles have a surprising organization; homologous chromosome pairs (bivalents) are ensheathed by microtubule bundles that run along their sides, whereas microtubule density is low at chromosome ends. Moreover, our previous studies revealed a distinctive feature of bivalent organization. We found that the chromokinesin KLP-19 is targeted to a ring around the center of each bivalent and provides a polar ejection force that is required for congression. The chromosome passenger complex (CPC) also localizes in mid-bivalent rings and is required for proper KLP-19 localization. These observations create a new picture of chromosome/microtubule association in acentrosomal spindles and reveal strategies that oocytes use to achieve chromosome alignment. We are now extending our findings by further analyzing the composition of the midbivalent rings and by investigating how chromosome segregation is accomplished in the context of this distinct organization. 

First, we find that the checkpoint protein BUB-1 (which has been shown by others to colocalize with both outer kinetochore and midbivalent proteins) forms a ring around the center of each bivalent. Localization of BUB-1 to the midbivalent rings (but not to the kinetochores) is regulated by the CPC. Proteins that form midbivalent rings leave the chromosomes in anaphase as the two homologs separate. Interestingly, while members of the CPC appear to associate broadly with the anaphase spindle, BUB-1 and KLP-19 persist in ring structures that remain in the center of the spindle, distant from the segregating chromosomes. Moreover, depletion of KLP-19 causes defects in spindle morphology during anaphase in some oocytes, raising the possibility that these rings may play a role in organizing microtubules at this stage. Current work is focused on testing this hypothesis and on analyzing the forces that drive chromosome segregation during meiosis.

**602/B549 ABSTRACT WITHDRAWN**

**603/B550 Conserved Male-Specific PP1 Phosphatases GSP-3 and GSP-4 Regulate Chromosome Segregation during Spermatogenesis in *C. elegans*.
J. Wu, D. S. Chu; Biology, San Francisco State University, San Francisco, CA**

Proper chromosome partitioning into each haploid sperm during meiosis is important for sperm function. Our lab has previously identified two PP1 phosphatases, GSP-3 and GSP-4, that are required for normal sperm formation in *C. elegans*. GSP-3/4 are 98% identical and are homologous to the mammalian male-specific PP1 phosphatases PP1γ. In mice, knock out of PP1γ results in male infertility. Consistent with the evolutionarily conserved function of these proteins, male worms lacking *gsp-3* and *gsp-4* are infertile and produce anuclear sperm or sperm with a single aberrant bar-like chromosomal mass, indicating defective chromosome segregation. Because GSP-3/4 localize to chromosomes undergoing division, we hypothesize that GSP-3/4 regulate the function of chromosomal proteins that are required for proper chromosome segregation. We have established a protocol that allows live imaging of male meiosis in *C. elegans*. Through live imaging, we found that in *gsp-3 gsp-4* mutant spermatocytes homologous chromosomes are separated at a speed comparable to the wild type. However, the second meiotic division is defective in the absence of *gsp-3* and *gsp-4*. During meiosis II in *gsp-3 gsp-4* mutant spermatocytes, the chromosomes align at the equator at metaphase II but fail to separate from each other when anaphase II starts, resulting in stretched chromosomes. Further cytological analyses show that *gsp-3 gsp-4* chromosome segregation defects do not originate from the failure to remove chromosomal cohesin between sister chromatids, a process facilitated by the aurora B kinase. We found that cohesin REC-8 is normally removed from *gsp-3 gsp-4* mutant spermatocytes. Moreover, the aurora B kinase AIR-2 is normally localized to the chromosomes in the absence of *gsp-3* and *gsp-4*. We are currently investigating if the chromosomes are correctly oriented to face the opposite spindle poles at metaphase II in *gsp-3 gsp-4* mutants. We are also testing if the kinetochore structure is defective in *gsp-3 gsp-4* mutants. These experiments will shed light on the male-specific modulation of molecular mechanism by male meiosis occurs.

**604/B551 The Role of Myosin Phosphorylation in Anaphase Chromosome Movement.**
This work deals with myosin function during cell division. Previous studies indicated that myosin might be involved in anaphase force production (Silverman-Gavrila and Forer, Cell Motil Cytoskeleton, 2003, 55:97; Fabian and Forer, Protoplasma, 2005, 225:169). Fabian et al. [Cell & Chromosome, 2007, 6:1] showed that CalyculinA (CalA), which blocks dephosphorylation of myosin, accelerates chromosome movement, presumably due to hyper-phosphorylation of myosin regulatory light chain (MRLC). They showed also that Y-27632, which inhibits myosin phosphorylation via the Rho-kinase pathway, slowed or stopped anaphase movements, consistent with the possible role of myosin in force production. CalA should not have reversed the effect of Y-27632, since the drug already blocked myosin phosphorylation, but it did. Fabian et al. therefore hypothesized that myosin can be phosphorylated by alternate pathways. We tested this by drug treatments using Y-27632, ML-7 and staurosporine which inhibit Rho-kinase, myosin regulatory light chain kinase and non-specific kinases respectively. Singly or triply all stopped or slowed chromosome movement. CalA did not reverse the effect of staurosporine treatment individually or in combination with Y-27632 and ML-7, consistent with myosin being phosphorylated by alternate pathways. To test the effects of the drugs on MRLC phosphorylation we studied treated cells using four different antibodies against phosphorylated MRLC. Chromosomal spindle fibres in control cells were stained with all antibodies, and there was reduced staining in cells treated with either staurosporine or all three drugs together. This is consistent with drugs causing slowed or stopped chromosome movement via effects on phosphorylation of spindle fibre myosin.

605/B552
Rapid Poleward Movement of Chromosomes Following Laser Microsurgery.
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The objective was to elucidate the roles of kinetochores of lagging X-Y chromosomes in (a) normal segregation during anaphase B and (b) their rapid poleward movement upon release from its bipolar attachments following laser microsurgery. For normal segregation, X and Y sex univalents in crane-fly spermatocytes each have two sister kinetochores, one attached to a leading kinetochore fiber that shortens and the other to a trailing fiber that elongates. Both leading and trailing kinetochores maintain a “polymerization” state during anaphase. This results in poleward microtubule flux in leading and trailing fibers as anaphase progresses, and thus shortening of leading fibers must occur at their polar ends. Flux was visualized directly via fluorescent speckle microscopy at velocities away from kinetochores of about 0.4μm/min. A laser microbeam was used either (a) to separate sisters or (b) to ablate trailing kinetochores. In either case, resultant poleward movement of a released leading kinetochore was both immediate and impressively more rapid than normal, ranging between 0.8 and 1.7μm/min, the latter being over four times the average flux rate mentioned above. To account for such rapid post-op movement, our interpretation is that following microsurgery, kinetochores may have shifted from a polymerization state (adding tubulin subunits to growing plus ends) to a depolymerization state (removing subunits from shrinking plus ends). After such a shift, leading kinetochore fibers could then shorten from both ends. If so, that leads us to conclude that kinetochores of univalents may indeed have the potential for “Pac-man” activity, but they appear to be maintained in a polymerization (“reverse Pac-man”) state by the resistive tension imparted by kinetochore fibers attached to trailing kinetochores. Upon detachment of a trailing fiber through microsurgery, such tension would be lost and the activation of kinetochore-based depolymerization (Pac-man) could occur.

606/B553
Aurora-C, but Not Aurora-B, Is Required for Meiosis in Mouse Oocytes.
The Aurora kinases play essential roles in many aspects of cell division including the control of centrosome function, kinetochore-microtubule interactions, and cytokinesis. In mammals, three distinct aurora kinase members (Aurora-A, -B, and -C) have been identified and all share similar structures at their central catalytic kinase domains. We previously isolated a novel kinase Aie1, now renamed as Aurora-C, in a screen for kinase expressed in mouse sperm and eggs (DNA Cell Biol. 17, 823-833, 1998). Aurora-C, a close homolog of Aurora-B, is enriched in testicular germ cells and appears to be essential for male mouse meiosis (Dev. Biol. 290, 398-410, 2006). However, the functions of Aurora-C in female meiosis have not been explored. Herein, we examined the roles of Aurora-C during female meiotic divisions in mouse oocytes. Our immunoblotting and immunofluorescence analysis revealed that endogenous Aurora-C kinase protein, but not Aurora-B, was detected in mouse oocytes. Aurora-C was found at the chromosome axes and centromeres at meiotic metaphase I and concentrated at centromeres at meiotic metaphase II. During the anaphase I/telophase I and anaphase II/telophase II transitions, Aurora-C was relocalized to the midzone and midbody. Microinjection of the kinase-deficient Aurora-C (AurC-KD) mRNA into mouse oocytes significantly inhibited endogenous Aurora-C activity in mouse oocytes and caused multiple defects, including lagging chromosomes, aberrant spindle formation, abnormal kinetochore-microtubule attachment, premature chromosome segregation, and interkinesis failure. Furthermore, microinjection of AurC-KD mRNA also reduced histone H3 phosphorylation, Aurora-C phosphorylation, and inhibited centromere/kinetochore localization of Bub1 and BubR1. Interestingly, oocytes with defective Aurora-C kinase activity accumulate kinetochore-microtubules in syntelic attachments and reveal premature segregated chromosomes (most were univalent). We thus propose that Aurora-C may function as a chromosomal passenger protein that regulates chromosome segregation, kinetochore-microtubule attachment and interkinesis during female meiosis in mammals.

607/B554
Molecular Distinctions between Mammalian Aurora A and B: A Single Residue Change Transforms Aurora a into Correctly Localized and Functional Aurora B.
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Mammalian Aurora A and Aurora B are paralogue mitotic kinases that share highly similar primary sequence, especially in their catalytic domains. Both are important to mitotic progression, but their localizations and functions are distinct. Aurora a associates with centrosomes and with centrosome proximal microtubules, and both binds to and is activated by TPX2, a microtubule binding protein that localizes Aurora a to the mitotic spindle. In contrast, Aurora B exhibits passenger protein behavior, first associating with the inner centromere, then separating from centromeres to relocate to the spindle midzone during cell cleavage. It binds to and is activated by INCENP, another passenger protein. Aurora B is absolutely required for spindle assembly checkpoint function, correct chromosome segregation and for cell cleavage. Despite their structural similarities, the two proteins have unique spectra of binding partners and of phosphorylation substrates. We have combined shRNA suppression with overexpression of Aurora mutants to address the cause of the distinction between Aurora a and Aurora B. Aurora a glycine residue G198, mutated to asparagine to mimic the aligned asparagine residue N142 of Aurora B, causes Aurora a to bind the Aurora B binding partner INCENP, but not the Aurora a binding partner TPX2. When Aurora B is ablated and replaced with G198N Aurora A, the Aurora a mutant rescues Aurora B mitotic function. We conclude that binding to INCENP is alone critical to the distinct function of Aurora B. Although G198 of Aurora a is required for TPX2 binding, N142G Aurora B retains INCENP binding and Aurora B function. Thus, while a single residue change transforms Aurora A, the reciprocal mutation of Aurora B does not create Aurora a
function. An Aurora A-delta120 N-terminal truncation construct reinforces Aurora A similarity to Aurora B, as it does not associate with centrosomes, but instead associates with kinetochores.

608/B555
An Enhanced Chemical Genetic Strategy to Allow Covalent Inhibition of the Fission Yeast Aurora Kinase.
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Analog-sensitive (as) kinases differ from their natural counterparts by a single amino-acid change in the ATP binding pocket. This so called gatekeeper mutation enlarges the ATP binding site and makes the kinase susceptible to specific inhibitors that typically do not target unmodified kinases. The high conservation of the kinase domain allows identification of the gatekeeper residue in most kinases, and as-kinases have been successfully created in organisms from yeast to human. Here we report on the introduction of a second mutation to successfully target a serine/threonine as-kinase with covalent inhibitors. Through structure-based design we identified a residue close to the ATP binding pocket, whose mutation to cysteine creates an anchor point for irreversible kinase inhibitors. We demonstrated the feasibility of this approach for an as-version of the conserved fission yeast (Schizosaccharomyces pombe) Aurora kinase Ark1. Aurora is essential for the proper execution of mitosis and small-molecule inhibitors selective for Aurora have been crucial to decipher its role during the cell cycle. We employed structure-guided chemical synthesis of a focused library of 4-anilino-quinazolines equipped with diverse reactive groups and show that some of the compounds specifically inhibit Ark1 carrying the gatekeeper mutation as well as the engineered cysteine, but not the kinase with only one of these mutations.

609/B556
Functional Characterisation of Newly Identified Protein-Interactions of the Cell Cycle Regulatory Kinase Aurora-A.
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Besides the centrosome’s classical function as the major microtubule organising center, it has also been implicated in cell cycle control and progression. Identification of cell cycle regulatory proteins at the centrosome strongly argues in favour of this model. Aurora-A is one of these proteins and functions in centrosome maturation. Centrosome maturation describes recruitment of additional proteins to the pericentriolar matrix to increase the microtubule nucleation capacity of the centrosome during mitosis. Furthermore, Aurora-A is involved in cell cycle regulation and is overexpressed e.g. in breast tumours and pancreatic tumours. To define substrate proteins of Aurora-A and the proteins that modulate Aurora-A activity during the cell cycle, we isolated Aurora-A interaction partners via Tandem Affinity Purification (TAP). Using mass spectrometry we identified a total number of 60 interactants. Among these proteins were cell cycle regulatory phosphatases and centrosomal proteins. We identified and confirmed the protein phosphatase 6 as one of the Aurora A interacting proteins. To analyze the functional relevance of this interaction we employed siRNA mediated RNAi in mammalian cells. Knock-down of the protein phosphatases catalytic unit leads to a change in the mitotic index implicating a role in cell cycle control. Using an RNAi approach we investigate the function of the fly homologues of Aurora-A and the protein phosphatases catalytic unit on a cellular and organism level. We demonstrated by immunoprecipitation that the interaction is conserved between human and the fly. Knock-down of aurora in SL2 cells strongly reduces the number of prophase cells with Microtubule Organising Centres (MTOCs) in a protein phosphatase 6 dependent manner. The functional and molecular characterisation of the interplay between the centrosomal kinase Aurora-A and its interacting proteins will contribute to further understanding of this kinases important role in cell cycle control, centrosome structure and microtubule organisation.

610/B557
Insights into the Role of Aurora B and Mps1 in the Spindle Assembly Checkpoint.
The Spindle Assembly Checkpoint (SAC) is a safety device that monitors microtubule-kinetochore attachment and delays anaphase onset until all sister chromatids have achieved bipolar attachment to the mitotic spindle. Protein phosphorylation by mitotic kinases is crucial for the SAC. Among the core components of the SAC, several proteins, including Aurora B, Bub1, BubR1, and Mps1, have a kinase domain. Here, we report the characterization of two checkpoint kinases, Aurora B and Mps1, using cell-permeable small molecules selectively targeting their kinase activities. Firstly, we describe the characterization of a new Mps1 inhibitor named Reversine as an inhibitor of the SAC. Importantly, Reversine treatment led to undetectable levels of Zwilch and Mad1 at unattached kinetochores, which is consistent with the powerful SAC override observed in the presence of this inhibitor. Intriguingly, we found that after a recovery from a STLC block (that leads to a monopolar spindle with several synthetical KT-MT attachments), Reversine-treated cells show a large number of mis-aligned chromosomes, indicating that besides its role in the SAC, Mps1 also plays a role in the correction of improper kinetochore-microtubule attachments. Aurora B was also implicated in error correction whereas its role in the SAC is controversial. Using the Aurora B inhibitor Hesperadin, we demonstrate that Aurora B, like Mps1, is strictly required for the SAC. While partial inhibition of Aurora B is still compatible with the checkpoint response to unattached kinetochores, complete ablation of its kinase activity leads to severe impairment of localization of SAC proteins at unattached kinetochores. Our findings suggest that a core component of the SAC, including the Aurora B and Mps1 kinases, responds to unattached as well as tensionless kinetochores. Thus, error correction is intimately intertwined with the SAC, establishing a new paradigm in the SAC.

**611/B558**
**FRET-Based RNAi Screen Identifies New Regulators of Aurora B.**
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Aurora B kinase controls various aspects of mitosis and cytokinesis. How Aurora B controls these different processes, particularly during late stages of cell division, is not fully understood. Using an Aurora B phosphorylation FRET-sensor targeted to chromatin (Fuller et al., Nature 2008), we here established live imaging-based RNAi screening for Aurora B regulators in human cells. We used supervised machine learning for automated annotation of mitotic stages to detect alterations in Aurora B phosphorylation. Within a siRNA library targeting genome-wide sets of phosphatases and kinases, we identified two phosphatases as new candidate regulators of Aurora B phosphorylation during anaphase. Ongoing experiments aim to validate and dissect their exact function.

**612/B559**
**Epigenetic Centromere Specification Directs Aurora B Accumulation at the Inner Centromere but Is Insufficient for Mitotic Error Correction.**
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The nearly ubiquitous presence of repetitive centromere DNA sequences across eukaryotic species is in paradoxical contrast to their apparent functional dispensability. Recent examples of intact human chromosome variants in which the centromere has moved to a new location lacking detectable α-satellite DNA provide prime examples of the epigenetic nature of centromere identity. Centromeric chromatin is spatially delineated into the kinetochore-forming array of CENP-A-containing nucleosomes and the inner centromeric heterochromatin that lacks CENP-A but recruits the Aurora B kinase that is necessary for correcting erroneous attachments to the
mitotic spindle. To investigate the structural features of centromeric chromatin required for its diverse functions in centromere segregation, we examined a patient-derived cell line containing a chromosome 4 variant that is intact in primary DNA sequence but has moved its centromere to a site that lacks repetitive DNA and is 25 Mbp away from the original, epigenetically silenced locus. We found that the self-perpetuating network of centromere proteins at the foundation of the kinetochore is intact at the neocentromere. Surprisingly, we found that the neocentromeric chromosome is >5-fold more likely than normal chromosomes to fail to correct spindle attachment errors, indicating that the Aurora B-dependent error correction mechanism is severely compromised at the neocentromere. Indeed, Aurora B is mis-positioned at the neocentromere at a site distal from its kinetochore targets. We monitored phosphorylation of a known Aurora B kinetochore target (Ser7 of CENP-A) in monopolar cells where spindle biorientation is impossible and Aurora B kinase activity at kinetochores is known to be high and found a >4-fold reduction in its kinase activity at the neocentromere compared to normal centromeres. Thus, the inner centromere architecture of the neocentromere is insufficient to support the robust error correction mechanisms present at centromeres with normal inner centromere geometry. Our data supports a model wherein a neocentromere represents a primordial inheritance locus that requires subsequent recruitment of repetitive DNA to enhance fidelity of chromosome transmission.

613/B560
Bod1 Regulates Mitotic Progression and Chromosome Segregation by Modulating Aurora B and Plk1 Activity.
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Biorientation defective 1 (Bod1) is a 22 kDa protein conserved across metazoans that we recently demonstrated was required for proper chromosome biorientation (Porter et al. J Cell Biol (2007)). Detailed examination of mal-oriented chromosomes in Bod1 depleted cells revealed a high incidence of syntelic attachments which we demonstrated was due, at least in part, to the failure of the Aurora B kinase to phosphorylate MCAK. Here we present data that demonstrates Bod1 also has a critical role modulating the function of Plk1 at centrosomes and kinetochores. After siRNA depletion of Bod1 Plk1 is partially delocalised from centrosomes and kinetochores during mitosis. Consistent with this γ-tubulin localisation was reduced ~3 fold at the centrosomes of Bod1 depleted cells. In addition, Plk1-dependent phosphorylation of the kinetochore constituent PBIP was also reduced after Bod1 depletion. Sgo1 and the splice variant sSgo1 localise to kinetochores and centrosomes, respectively, preventing precocious separation of sister chromatids and mother and daughter centrioles prior to anaphase onset. Sgo1 is required, at least partially, to localise Plk1 to kinetochores and phosphorylation of sSgo1 by Plk1 is required for its localisation to centrosomes, suggesting a reciprocal requirement for localisation of these proteins to kinetochores and centrosomes. Depletion of Bod1 reduced localisation of Sgo1 and sSgo1 to kinetochores and centrosomes, respectively. Consistent with this result, we observed precocious separation of sister chromatids in fixed and living cells. Other factors implicated in the localisation of Sgo1, such as Bub1, Sgo2 and PP2A all localise properly in Bod1 depleted cells suggesting that the failure of Sgo1 to localise properly is a result of delocalisation of Plk1. Finally, we also show that in the absence of Bod1 Plk1 is more sensitive to the small molecule inhibitor BI 2536 and that mitotic entry is also slowed suggesting that Bod1 is also required to modulate Plk1 activity during mitotic entry. These data, in combination with our previous work indicate that Bod1 modulates the activity of both Aurora B and Plk1 kinases and thus is a critical regulator of mitotic progression and chromosome segregation.

614/B561
Two Kinesin-Like Motors Spatially Regulate Chromosome Movements during Mitosis.
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During mitosis in vertebrate cells, chromosomes oscillate with periods of smooth motion interrupted by abrupt reversals in direction. The molecular mechanisms that spatially constrain
these movements to facilitate the alignment and segregation of chromosomes with high fidelity are not well understood. Through spatially resolved analyses of chromosome movements in live cells, we have identified a strong correlation between the rate of directional switches and chromosome position within the spindle. Our studies indicate that two kinesin-like motors, Kif18A and Kid, synergistically provide this spatial regulation and promote congression. Depletion of Kif18A or Kid produces chromosome alignment defects and a reduced dependence of switch rate on position within the spindle. Co-depletion of Kif18A and Kid further increases the severity of these phenotypes. While these motors similarly affect chromosome positioning, Kif18A and Kid have opposite effects on the directional switch rate and velocity of chromosome movement. In cells depleted of both kinesins, these effects are additive such that kinetochore movement parameters are similar to those measured in control cells despite the severe disruption of chromosome alignment seen under these conditions. These data indicate that two kinesin-dependent mechanisms act in parallel to provide position-dependent regulation of chromosome movement and facilitate alignment at the spindle equator.

615/B562
A Countdown Clock in Mitotic Prophase.
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Mitotic progression is controlled by a set of clocks and dominoes that time key mechanical events and ensure accurate chromosome segregation. It is currently unknown how the earliest mechanical events of mitosis, centrosome separation and nuclear envelope breakdown (NEBD), are timed. Here we track centrosome separation using high-resolution time-lapse imaging of HeLa cells that express Eg5-mEYFP and mCherry-α-tubulin. We show that the rapid loading of Eg5 motor onto centrosomes at the outset of prophase indexes the early mechanical events of mitosis relative to a countdown clock that controls NEBD. Following Eg5 loading this clock sanctions a 9-minute time window during which the cell attempts to separate the centrosomes using Eg5-driven antiparallel microtubule sliding. 1µM EII, an Eg5 specific inhibitor, delays centrosome separation whilst leaving the NEBD-clock intact. After 9 minutes, NEBD ensues, whether or not centrosome separation has completed. Our results reveal the design logic of mitotic prophase: it makes sense to spend the time sanctioned by the NEBD-clock attempting to drive the incipient spindle poles to opposite sides of the DNA, because, as we show, this speeds up the subsequent steps of bipolar spindle assembly by ~3 minutes. but there is no point in delaying NEBD, because following NEBD, spindle assembly can, in any case, proceed by alternative pathways that incur only a slight time penalty.

616/B563
P38 Activity Is Required for Timely Satisfaction of the Mitotic Checkpoint but Not for Normal Chromatid Segregation.
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p38 mitogen-activated protein (MAP) kinase is activated in response to various stresses but its role, if any, during mitosis is unclear. We found that activated (phosphorylated) p38 weakly associates with the centrosome during G1 and with the oldest centrosome during S and G2. However, near the time of nuclear envelope breakdown its activity significantly increases and it becomes associated both with the new (daughter) centrosome and to a lesser extent with each half-spindle. Active p38 also strongly targets the centrosomes in nocodazole-treated mitotic cells lacking microtubules, and during the mitotic block its activity slowly diminishes until it is reduced by ~95% after 16 hrs. Thus active p38 is a bona-fide component of the mitotic centrosome. To determine whether its activity is required during mitosis for normal spindle function we asked how the duration of mitosis (DM) is affected in RPE-1 cells after inhibiting p38 with small molecule inhibitors (SB2035580 or SB202190) or RNAi knockdown. We found that although under these conditions chromatid segregation and cytokinesis was normal, the DM was prolonged by ~50%. To determine if the upstream p38 activator (MKK3/6) or downstream target (MK2) of p38 are also required for timely progression through mitosis we studied the DM in MEF cells lacking MKK3/6.
and in RPE-1 after MK2 RNAi. We found that DM was also significantly prolonged in both the former (≈25%) and latter (≈50%) cells. Finally, we found that the prolongation of mitosis in cells lacking p38 activity occurs selectively during prometaphase and, as a result inhibiting p38 leads to an accumulation of prometaphase cells. We propose that p38 activity is required for the timely stable attachment of all kinetochores to the spindle, i.e., satisfaction of the mitotic checkpoint, and not for normal APC or proteasome activity.

617/B564
An Automated Method for Multi-Object Tracking in Crowded Environments.
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Recent advances in novel imaging techniques have sparked an exciting new era for cell biology. However, extracting the biologically relevant information from these data remains challenging, and the problem is often tackled by laborious manual tracking of objects in the fluorescence images. Here we outline a novel algorithm for automated object tracking in crowded environments with single particle resolution. The method utilizes two-stage cross-correlation based template matching and is shown to be efficient on data from a broad range of systems. The algorithm has been applied to track individual kinetochores during Anaphase in human U2OS cells, and to uncover the precise dynamical behavior of growing microtubule (MT) plus-ends in Drosophila S2 cells. In the latter, an extremely unexpected result was discovered, namely that MT growth slows down in cells lacking a particular mitotic protein, KLP59D. This result is surprising because KLP59D is known to be responsible for depolymerizing MTs and is a member of the Kinesin-13 family of MT depolymerases. In addition the algorithm has also been used to follow centrosomes during FRAP assays during which the centrosomes were seen to move appreciably, thus requiring tracking for more accurate fluorescence recovery data by removing a systematic component of the noise in the signal.

618/B565
A Critical Role of PRC1 in Controlling Microtubule Assembly and Dynamics That Regulate Spindle Formation and Integrity in Mitosis.
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Microtubule-associated proteins (MAPs) play critical roles in controlling microtubule (MT) assembly and dynamics especially during spindle formation. However, the mechanisms by which MAPs regulate these complex processes are poorly understood. Here, we use Xenopus egg extracts to reveal a new function for the mitotic MAP, protein regulator of cytokinesis 1 (PRC1), in controlling MT assembly and dynamics, thereby regulating spindle formation and integrity in mitosis/meiosis. Perturbation of XPRC1 (Xenopus PRC1) function inhibits MT assembly and formation of normal size asters and spindles. XPRC1 inhibition also causes reductions in aster/spindle density and the efficiency of bipolar spindle formation. Although XPRC1 is not an essential factor for Ran-dependent MT assembly, XPRC1 functions as a general MT stabilizing protein that acts synergistically with the MT polymerase XMAP215 and antagonistically with the MT depolymerase XKCM1 to regulate MT assembly. Time-lapse microscopy reveals that XPRC1 controls MT assembly and dynamics by reducing the MT shrinkage rate and increasing the MT nucleation frequency. Inhibition of XPRC1 function in anaphase spindles severely inhibits spindle midzone formation, resulting in MT depolymerization, spindle shrinkage and ultimately disintegration. Together, our results demonstrate that, besides its well-characterized anti-parallel MT bundling activity, PRC1 also controls MT assembly and dynamics to modulate spindle formation and maintain spindle integrity in mitosis/meiosis.

619/B566
Dissecting the Roles of Ndel1 N-Terminal Binding Partners in Microtubule Self-Organization.
The cytoplasmic dynein motor complex is involved in a variety of cellular processes. Nde1 and Nde1 are well characterized regulators of dynein. We find that the Nde1 protein is developmentally regulated and is detected at very low levels in CSF-arrested Xenopus extracts. Nde1, on the other hand, is present in Xenopus egg extracts and involved in the 'self-organization' of microtubules into asters, which is a dynein-dependent process. Interestingly, the N-terminal coiled-coil of Nde1 is sufficient to carry out this function of Nde1, demonstrating that the N-terminus of Nde1 constitutes its functional domain. We propose that the C-terminal unstructured tail inhibits this active domain, until the proper signals are generated. We have mapped Nde1 interactions with other proteins and tested their requirements to focus microtubules. First of all, the presence of the Lis1 binding domain within residues 100-160 is critical. Second of all, we have identified a novel dynein-interacting site in the N-terminus of Nde1. Truncated Nde1 proteins, which do not have this domain, do not rescue aster formation in Nde1 depleted extracts. We took a closer look at Nde1 interactions with Lis1 and dynein. We show that Lis1 binding is critical for the Nde1 function in extracts. In addition, Lis1 is required for proper function of microtubules independent of Nde1. We have also mapped the dynein interacting domain within the coiled-coil of Nde1. Together, our data suggest that the N-terminus of Nde1 acts as a structural scaffold bringing cytoplasmic dynein together with its regulators.

620/B567
Identification and Characterization of a Novel Kinesin-Like Protein (KifX) Necessary for Bipolar Spindle Assembly.
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During mitosis chromosome segregation relies on the proper formation and stabilization of the mitotic spindle. Although many proteins have been identified that contribute to spindle formation, presumably many others are involved. To identify novel proteins involved in mitotic spindle formation, mitotic HeLa cell extracts were used for in vitro microtubule polymerization reactions and proteins co-pelleting with microtubules were identified by mass spectrometry. We generated a Dharmacon siRNA library directed against 600 microtubule binding proteins for use in high throughput genetic screens, including mitotic spindle assembly, mitotic arrest, mitotic bypass, and apoptosis. Among the positive hits from these screens was a novel kinesin-like protein termed KifX. KifX is ubiquitously expressed at low levels among all tissues examined, including skin, brain, and spinal cord. KifX localizes to the centrosome and more specifically to the centrioles in a cell cycle specific manner, from prophase to anaphase. Depletion of KifX resulted in multipolar spindle formation, however no centrosome over-duplication was observed. KifX-depleted cells exhibit pericentriolar material (PCM) fragmentation, indicated by multiple Pericentrin and gamma-Tubulin foci, but only two centrosomes by Centrin staining. Moreover, this defect can be rescued by inhibition of the mitotic kinesin Eg5 or the mitotic kinase PLK1 prior to mitotic entry. This implicates KifX as having a role in early mitosis, post centrosome separation, which is required to keep the PCM intact. Localization of spindle focusing activities (TPX2, Eg5, NUMA, etc) to spindle poles is not affected in KifX-depleted cells as these factors localized to ectopic spindles. Live time-lapse microscopy shows that KifX-depleted cells enter mitosis but fail to align chromosomes at the metaphase plate. These cells do not exit mitosis, condense their chromatin, and apoptose in mitosis. KifX-depleted cells have increased caspase 3/9 activity along with DNA fragmentation and decreased cell viability. KifX represents a novel factor in spindle formation and may help to elucidate the requirements for triggering mitotic apoptosis, sought after in antimitotic therapeutics.

621/B568
Analysis of Mph1 Kinase and Its Substrates in Spindle Checkpoint Signalling.
Accurate chromosome segregation is crucial as mis-segregation results in aneuploidy and this can result in severe diseases such as cancer. The spindle checkpoint monitors sister-chromatid attachment to the mitotic spindle and inhibits the onset of anaphase until all chromosomes are correctly bi-oriented on the mitotic spindle. The anaphase promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase that drives cells into anaphase by targeting the separase inhibitor securin and cyclin B for degradation by the 26 S proteasome. Upon spindle checkpoint activation the APC/C is inhibited by the mitotic checkpoint complex (MCC) composed of Slp1/Cdc20, Mad2 and Mad3. The spindle checkpoint machinery of \textit{S. pombe} is composed of many proteins, one of which is the kinase Mph1 (Mps1p-like \textit{pombe} homolog). It previously has been shown that Mph1 is essential for the spindle checkpoint (He et al., 1998) but not whether this is due to its kinase activity. The aim of this study is to determine whether Mph1 kinase activity is required for the spindle checkpoint and to test whether any of the known spindle checkpoint proteins are Mph1 substrates. Furthermore, we wanted to test whether Mph1 has a regulatory role in MCC binding to the APC/C. Using a kinase-dead allele of Mph1, that had no detectable \textit{In Vitro} kinase activity, we showed that lack of Mph1 kinase activity abolished the spindle checkpoint and led to chromosome mis-segregation. In addition, lack of Mph1 kinase activity severely impaired Mad2 and Mad3 binding to the APC/C. Using \textit{In Vitro} kinase assays, Mad2 and Mad3 were identified as \textit{In Vitro} substrates of Mph1. Phosphorylation sites in Mad2 were determined by mass spectrometry. A phosphorylation mutant of Mad2 with 5 Mph1 sites replaced with alanine showed reduced APC/C binding and spindle checkpoint defects, indicating that it is an important Mph1 substrate.

622/B569

\textbf{Lamin B Restrains the Activity of Kinesin Eg5 in Spindles Assembled in Xenopus Egg Extracts.}

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Lamin B3 (LB3) is a structural component of the membranous mitotic spindle matrix and is important for proper spindle morphogenesis. However, how LB3 regulates spindle assembly remains unclear. We have developed assays in Xenopus egg extracts to study whether LB3 could regulate spindle assembly by balancing forces generated by the microtubule (MT)-based motors. We report here that LB3 antagonizes Eg5 kinesin activity in spindles stimulated by either Aurora a (AurA) beads plus RanGTP or sperm chromatin. LB3 inhibits Eg5’s ability to drive the separation of AurA beads that mark MT organizing centers (MTOCs) and spindle poles. Similarly, LB3 restrains Eg5 activity to help in maintaining spindle length and spindle pole focusing in spindles assembled from sperm chromatin. Since previous studies have shown that dynein and LB3 coordinate in the spindle matrix through interactions between Nudel and LB3, our studies provide evidence that LB3 restrains the forces produced by Eg5 during spindle morphogenesis as part of the spindle matrix.

623/B570

\textbf{Kinesin-8 Kif18A Regulates Spindle Length in a K-Fiber Independent Mechanism Separate from Its Role in Chromosome Congression.}

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During mitosis the cell assembles the mitotic spindle, a macromolecular structure composed of numerous proteins, which is utilized to segregate the chromosomes. The spindle microtubules (MTs) are highly dynamic polymers that are constantly undergoing polymerization and depolymerization within the spindle, and yet the spindle apparatus maintains a constant length
during mitosis. There are many molecules proposed to regulate its length, but how these molecules coordinate to maintain an average spindle length is currently unknown. Kinesins are a superfamily of molecular motor proteins that play multiple roles during mitosis, including regulating MT dynamics, spindle assembly, and chromosome segregation. We previously showed that the minus-end directed Kinesin-14 HSET regulates spindle length through its ability to crosslink and slide MTs of the spindle in a kinetochore fiber (K-fiber) independent pathway. Overexpression of HSET results in long bipolar spindles, whereas knockdown produces shorter spindles. The Kinesin-8 Kif18A is a plus-end directed motor and MT depolymerase that is important for chromosome alignment and for regulating chromosome oscillations. Knockdown of Kif18A causes chromosome misalignment and also results in abnormally long bipolar spindles, similar to those observed in HSET overexpression. Here we show that knockdown of both HSET and Kif18A rescues bipolar spindle length, however chromosome alignment remains perturbed. These results suggest that Kif18A has two distinct and independent roles in mitosis to regulate spindle length and to congress chromosomes. In addition, we show that the MT depolymerization activity of Kif18A is not restricted to K-fibers as proposed. Knockdown of Kif18A in combination with knockdown of the Ndc80 component hNuf2 results in longer spindles compared to Kif18A and hNuf2 single knockdowns, suggesting that Kif18A also acts on the more dynamic spindle MTs. Together these results suggest that 1) Kif18A regulates chromosome congression and spindle length through two separable pathways; and 2) Kif18A and HSET may coordinate within a K-fiber independent pathway to maintain an average spindle length through regulation of MT depolymerization and MT sliding.

624/B571
Promoting Error-Free Mitosis: Regulation of the Kinesin-13 Kif2b through Phosphorylation.
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Accurate chromosome segregation during mitosis is an essential process mediated by the microtubule-based spindle. To achieve proper segregation, kinetochores of sister chromatids must attach to microtubules extending from opposite poles. A common cause of chromosome mis-segregation is the persistence of improper attachments between kinetochores and spindle microtubules. The kinesin-13 Kif2b is required for the correction of these improper kinetochore-microtubule attachments (Bakhoum et al, 2008). Kif2b localizes to kinetochores in prometaphase where it promotes the correction of attachment errors through its microtubule destabilizing activity. We propose that the spatially and temporally-specific nature of Kif2b activity is regulated by unique phosphorylation events. Consistent with this proposal, localization of a GFP-Kif2b fusion protein to kinetochores is abolished by treatment with the Aurora kinase inhibitor Hesperadin. Conversely, the fluorescence intensity of GFP-Kif2b at kinetochores is significantly increased with treatment with the Polo kinase inhibitor BI-2536. To identify specific sites regulated by phosphorylation, we immunoprecipitated GFP-Kif2b from stably-expressing U2OS cells synchronized in prometaphase and metaphase as well as cells in interphase. Using mass spectrometry we identified 13 residues specifically phosphorylated in mitosis, three of which are phosphorylated only in metaphase. To determine which of these are the critical regulatory sites, we are systematically mutagenizing each residue, both individually and in combination, and determining the ability of each mutant to localize to kinetochores and correct kinetochore-microtubule attachment errors.

625/B572
Phosphorylation of the Kinesin-13 MCAK Regulates a Conformational Change between the Neck and C-Terminal Domain.
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MCAK is a Kinesin-13 that uses the energy of ATP hydrolysis to disassemble MTs and regulate MT dynamics. MCAK MT depolymerization activity is inhibited by phosphorylation at a single residue (S196) within the positively charged neck; however, the biochemical mechanism by which this phosphorylation regulates depolymerization activity is unknown. The activity of MCAK is also modulated by its C-terminal domain (CT), although this domain is not essential for activity. These findings imply that there is an interplay between the neck and CT that might regulate MCAK activity. We propose that the neck and CT physically interact and that phosphorylation regulates this interaction to modulate MT depolymerization activity. To test this model we performed In Vitro pull-down assays with GST-tagged CT and MCAK proteins containing various combinations of the N-terminal domain (NT), neck, and motor domains. We found that the CT interacted best with the construct containing only the neck and motor domain. Furthermore, Aurora B phosphorylation of the neck+motor construct resulted in significantly decreased binding to the CT that was due to phosphorylation at S196. To assess whether the regulated interaction between the CT and the neck exists in the context of full-length protein, we engineered fluorescence resonance energy transfer (FRET) biosensors with mCitrine on the N-terminus and mCerulean on the C-terminus of MCAK. We hypothesized that biosensors containing the CT of MCAK would display a high FRET ratio, whereas biosensors not containing the CT would display a low FRET ratio. Indeed, full-length MCAK displayed high FRET, and MCAK missing the CT displayed significantly reduced FRET. Interestingly, MCAK missing the NT, but containing the neck, motor, and CT domains displayed higher FRET than full-length MCAK, consistent with our binding data and suggesting that the neck is the important domain for the interaction. Phosphorylation of the biosensors by Aurora B significantly reduced the FRET ratio, suggesting that phosphorylation opens up the conformation of MCAK. Together these data indicate that MCAK is active in a closed state, but is inactive in an open state, and that phosphorylation regulates this conformational change.

626/B573

Roles of Drosophila Kinesin-13s in the Regulation of Spindle Microtubule Dynamics.

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Microtubule destabilizing kinesin-13s are important regulators of microtubule (MT) dynamics. Interestingly, many systems require the activities of multiple distinct kinesin-13 family members to successfully complete mitosis: for example, mitosis in Drosophila involves the activities of three kinesin-13s, KLP10A, KLP59C and KLP59D. The specific individual and collective influence of these proteins on the dynamic behaviors of distinct spindle MT subsets, particularly non-kinetochore microtubules, remains relatively unexplored to elucidate the effects of the Drosophila Kinesin-13s on non-kinetochore MTs we tracked trajectories of GFP-EB1, which tracks polymerizing MT plus-ends, in living cells depleted of the proteins individually and in combination by dsRNAi, using a novel in-house tracking algorithm. Our data indicate some intriguing and unexpected Kinesin-13 functions, for example, KLP10A and KLP59D, which both localize to mitotic centrosomes, strongly but distinctly alter astral MT behaviors. Depletion of KLP59D decreases astral MT growth rate, resulting in shorter astral arrays. Depletion of KLP10A, on the other hand, results in elongated astral MTs by inhibiting rescue events. This latter finding indicates a difference between KLP10A’s mitotic and interphase activities (in interphase, KLP10A promotes plus-end catastrophes). The roles of KLP59C are currently under investigation. Together, our data expand and specify the roles of distinct kinesin-13s in mitosis.

627/B574

The Drosophila Kinesin-13, KLP10a, Transits from Centrosomes to Spindle Poles.

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The Drosophila kinesin-13, KLP10A, performs multiple roles in mitosis ranging from the regulation of astral microtubule dynamics to the stimulation of poleward flux and poleward chromosome movements. To a large measure, this functional diversity can be attributed to KLP10A’s complex localization pattern: KLP10A targets mitotic centrosomes, spindle poles, microtubule tips and
centromeres/kinetochores. Here, we focus on the cellular dynamics of this kinesin-13 using FRAP and photoactivation to follow the movement and turnover of GFP-KLP10A and PA-GFP-KLP10A, respectively, to and from these primary sites of action. These analyses have revealed KLP10A to be extremely dynamic and move rapidly throughout the spindle. This is particularly so at centrosomes: the motile fraction of centrosomal KLP10A turnover with a t1/2 of 2.5 sec. Interestingly, the localization and turnover of KLP10A at centrosomes is regulated by a second kinesin-13, KLP59D. We have also used a variety of approaches to follow the movement of KLP10A from centrosomes and found a directional flow to spindle poles. Indeed, the presence of centrosomes in the cell, but not their association with the spindle, is required for the cumulation of normal levels of spindle pole-associated KLP10A. KLP10A induced depolymerization of microtubule minus-ends at spindle poles is believed to be a primary mechanism for stimulating poleward flux. We propose a model wherein passage of KLP10A through centrosomes activates its spindle pole activity.

**Cytokinesis I (628 – 644)**

**628/B575**

**Investigating the Role of Lipids in Cytokinesis.**

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Cytokinesis is the last phase of cell division, where cells physically separate. It involves the distribution of membranes, the cytoskeleton, organelles and proteins into two daughter cells. Understanding the mechanism of cytokinesis is a fundamental question in cell biology, which also has clinical relevance since failed cytokinesis has been linked to tumorigenesis. Although cells undergo dramatic shape changes during cytokinesis, the role of the plasma membrane and lipids is poorly understood. It is known that a few lipids localize to the cleavage furrow and that the specific distribution of these lipids is necessary for cleavage. However, the identity of other lipids that may be involved in cytokinesis, their precise function, how they are transported to the cleavage furrow and maintained there are still unclear. Hence, to identify further lipids that are involved in cytokinesis, and to develop tools to study these lipids, we perturbed the activity of enzymes that play a part in lipid biosynthesis and determined if this has an effect on cytokinesis. Specifically, we identified known small molecule regulators of lipid metabolism, and in particular targeting fatty acid, sphingolipid or cholesterol biosynthesis. We tested these compounds in HeLa cells and observed if incubation with these compounds caused cytokinesis failure, using both fixed and live imaging. We identified several regulators of lipid biosynthesis that induce the formation of binucleated cells. We are currently investigating the mode of action that results in cytokinetic failure and individual lipids that are enhanced/depleted due to compound treatments.

**629/B576**

**Sweeping of Actin into the Incipient Contractile Ring by Dynamic Microtubules.**

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It is known that contractile ring assembly involves interactions between spindle microtubules and the cell cortex. The nature of such interactions, however, remains to be determined. Here, we have directly probed the interactions between peripheral spindle microtubules and cortical actin filaments by micromanipulation in living grasshopper spermatocytes. Microtubule-actin interactions were demonstrated by pushing on the spindle or asters with a micromanipulation needle while rapidly imaging the interactions using a spinning disc confocal microscope equipped with an EM-CCD digital camera. When microtubules were tugged with a microneedle, the actin filaments at the cortex were concurrently dragged inward, causing the cell membrane to invaginate. The membrane relaxed once the needle was released. These interactions were first apparent during late anaphase and continued throughout assembly of the contractile ring. We also tested the interactions in the presence of cytochalasin D, which disassembled actin filaments.
into small aggregates that were mobile and readily visible. These cortical actin aggregates could ride on the tips of dynamic microtubules that were waving within and/or elongating towards the cortex. In light of our recent findings in silkworm spermatocytes (Chen et al., 2008, PLoS Biology), these results demonstrate a mechanism by which peripheral spindle microtubules could mechanically sweep cortical actin filaments into the incipient contractile ring.

630/B577
A Quantitative Study of Cell Shape and Spindle Orientation in Sea Urchin Embryos.

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The proper orientation of the mitotic spindle is critical for placement of the division plane for cytokinesis, cell shape, tissue architecture and development. Although several molecular aspects of spindle and nuclear positioning have been identified, in general little is known about the larger question of how cells sense their cell shape at a global level. Here, we investigated the effects of cell geometry in the sea urchin zygote, an otherwise symmetric cell that lacks inherent polarity cues. To alter geometrical parameters in a systematic and quantitative manner, we produced cells of different shapes (triangles, rectangles, squares and cones) by placing them into microfabricated PDMS chambers of different shapes and observing them using medium-throughput time-lapse imaging. Our results show that the spindles position along the long axis of the cell, and centers by somehow sensing cell volume, rather than cell length or surface area. The cleavage furrow forms in a plane perpendicular to the spindle regardless of distances between the cortical sites and the spindle. This approach associated with theoretical modeling will allow us to test and develop models for spindle positioning in mitosis and the division plane positioning in cytokinesis.

631/B578
CaM Has Distinct Role on Plk1’s Function during Cytokinesis Initiation and Completion.

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Objective: It has demonstrated that Plk1 activation is essential not only for the initiation of cytokinesis but also for successful furrow ingression once constriction has commenced, even for intracellular bridge abscission. Although the downstream targets of Plk1 have been dedicated examined, only few upstream regulator has been explored. Here we will confirm that CaM might be such a molecule that control Plk1’s activity and localization in a spatial-temporal dependent manner. Methods: We used FRET method to examine the In Vivo interaction between CaM and Plk1, applied chromophore-assisted laser inactivation (CALI) technique to acute inactivate CaM after cytokinesis onset, and during intracellular bridge stage. Results: We found that CaM interacted with Plk1 during early anaphase while fell apart during intracellular bridge stage. Kinase activity assay also showed that CaM could enhance Plk1’s activity during anaphase while inhibit Plk1’s activity during intracellular bridge stage. When we acute inactivated CaM with CALI technique after cytokinesis onset, the CaM-Plk1 interaction was broken and Plk1’s activity was diminished. Meanwhile, the cleavage furrow ingression was blocked and the mitosis exit was also stopped. The result indicates that the interaction between CaM and Plk1 contributes to the cleavage furrow ingression. but the situation was totally different during intracellular bridge stage when CaM has been separated from Plk1 at midbody. Although CaM inactivation blocked intracellular bridge abscission, even resulting in a binucleate cell (50% of observed cells), Plk1 inactivation accelerated intracellular bridge abscission. Plk1 overexpression could prolong intracellular bridge abscission. Conclusions: The results suggest that the spatiotemporal interaction between CaM and Plk1 plays different roles at different cytokinesis stages. CaM can positive regulate Plk1’s activity through direct physical interaction during anaphase, while decrease Plk1’s activity through a negative mediator when CaM and Plk1 are spatially separated during intracellular bridge stage.

632/B579
Differential Midbody Fate between Neural Stem Cells and Cancer-Derived Cell Lines.

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During mouse neurogenesis, neuroepithelial progenitor cells release midbodies while switching from proliferative to differentiative divisions. In order to get insight into this cell biological process, we studied midbody release in the neural stem cell line NS-5. To assay for released midbodies, we subjected conditioned culture medium of NS-5 cells and various cancer-derived cell lines to differential centrifugation followed by immunoblotting for two midbody markers, citron rho-interacting kinase (CRIK) and mitotic kinesin-like protein 1 (MKLP1). Strikingly, the extent of midbody release was significantly greater from NS-5 cells than from cancer-derived cells, and was increased upon differentiation of NS-5 cells. Interestingly, inhibition of autophagy by chloroquine or ammonium chloride increased persistence of midbody markers in HeLa, but not NS-5, cells. Thus, our results suggest that cultured neural stem cells can be used as a cellular model for midbody release. Moreover, the differential fate of the midbody between neural stem cells and cancer-derived cell lines suggests that there are two principally different routes of midbody disposal after cell division; (i) the transient retention of midbody constituents in cells, as exemplified in HeLa cells, and (ii) the release of midbody constituents from cells, as exemplified in neural stem cells. We propose that midbody release may be involved in cell differentiation, and that, conversely, midbody retention may contribute to the transformation of stem cells into cancer cells.

633/B580
Mitotic Regulation of Septin 9 Is Important for the Completion of Cytokinesis.

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Proper cell division is essential for multicellular development, and defects in this process have been linked to cancer. Septin 9 (SEPT9) is a GTP-binding protein that has been implicated in cell division, and linked to a variety of cancers including breast, ovarian and leukemia. In addition, a SEPT9 DNA methylation assay was recently developed that allows sensitive detection of colorectal cancer, further linking SEPT9 expression to cancer. However, the precise role of SEPT9 during cell division and its mechanism of regulation during this process remain elusive. Here we demonstrate that SEPT9 is phosphorylated upon mitotic entry, which controls association with the proline isomerase, Pin1. Surprisingly, we find that SEPT9 is dispensable for the early stages of cell division, but both SEPT9 and Pin1 are critical for mediating the final separation of daughter cells. While wild type SEPT9 expression rescued the cytokinetic defect seen upon SEPT9 depletion, expression of mutant SEPT9 that is defective in Pin1 binding or a SEPT9 isoform that lacks the Pin1 binding site led to severe defects in cytokinesis. These results suggest that mitotic regulation of SEPT9 is important for the completion of cytokinesis.

634/B581
Centralspindlin and the Chromosomal Passenger Complex Coordinate the Promotion of Furrow Ingression in the C. elegans Embryo.

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Cytokinesis is the final step of mitosis that physically divides a single cell into two daughter cells following chromosome segregation. Although it is known that the anaphase spindle is continuously required for cytokinesis, the precise signaling mechanisms that promote formation and constriction of the contractile ring are not known. The spindle midzone, which is an array of antiparallel microtubule bundles that forms during anaphase, serves as a scaffold for the

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localization of two key complexes that are required for cytokinesis in all systems - Centralspindlin and the Chromosomal Passenger Complex. Here, we use the C. elegans embryo to dissect the contributions of each complex during cytokinesis.

635/B582
**A Novel Coiled-Coil Protein Is Required for the Localization of Plk1 to the Central Spindle.**
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Polo-like kinase 1 (Plk1) localizes to the central spindle and plays a central role in regulating furrow formation and ingresssion. However, it is still not completely understood how Plk1 is concentrated to the central spindle. Here we report that a novel coiled-coil protein CCDC69 is required for the localization of Plk1 to the central spindle. Northern blot and immunoblot analysis show that CCDC69 is ubiquitously expressed in multiple human tissues and numerous human cancer cell lines. Immunofluorescence analysis shows that endogenous CCDC69 localizes to the nucleus during interphase and to the central spindle during anaphase. GST pull-down assays show that CCDC69 can physically interact with Plk1. Further, immunofluorescence analysis shows that CCDC69 and Plk1 colocalize to the central spindle and midbody during mitosis and cytokinesis. Depletion of CCDC69 by RNA interference (RNAi) disrupts the localization of Plk1 to the central spindle. Our results suggest that CCDC69 may play a role in localizing Plk1 to the central spindle.

636/B583
**Analysis of Pathways Required for Nuclear Envelope Disassembly.**
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The nuclear envelope (NE) is a double-membrane sheet that is contiguous with the membranes of the endoplasmic reticulum (ER). Metazoans must disassemble the NE prior to chromosome segregation. In the C. elegans zygote, after the two pronuclei meet, the nuclear envelopes are permeabilized and cleared from the region near the centrosomes, promoting spindle assembly. After chromosome segregation, a single nuclear envelope reforms in each daughter cell, thereby mixing the haploid sperm and oocyte genomes. In embryos in which NE disassembly is partially inhibited, separate nuclear envelopes form around the sperm- and oocyte-derived chromosomes after their segregation, resulting in two nuclei in each daughter cell. Functional genomic analysis of cell division in the C elegans embryo uncovered relatively few proteins whose depletion caused the "four-eyes" phenotype suggesting that other proteins important for NE disassembly were missed or that redundant pathways exist. We identified a novel pathway that contributes to removal of nuclear membranes prior to completion of cytokinesis. We suggest that NE disassembly and cytokinesis are spatially and temporally coupled so that each daughter cell contains a single membrane-enclosed nucleus.

637/B584
**Contractile Ring Asymmetry Is Regulated by Anillin Protein Level during Cytokinesis in C. elegans.**
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Anillin is a conserved cytoskeletal cross-linker that is important for cytokinesis, and localizes to the contractile ring with its structural binding partners: actin, myosin and septins. In the C. elegans early embryo, when Anillin is depleted by RNA-mediated interference, cytokinesis can occur, but the contractile ring displays several abnormalities, including circumferential symmetry. We are studying Anillin using C. elegans strains stably expressing GFP-tagged transgenes. Interestingly, our four different GFP-tagged full-length Anillin constructs are expressed at different basal levels and respond differently to depletion of endogenous Anillin. This could be due to the stochastic nature of transgene integration. Expression of each GFP-tagged Anillin can rescue
embryonic lethality caused by Anillin depletion, but none of the transgenes can fully replace function of endogenous Anillin in contractile ring asymmetry. To understand how Anillin protein level impacts ring asymmetry, we performed a depletion time-course, measuring Anillin levels by western blotting and monitoring asymmetry by live cell imaging of embryos expressing GFP-tagged myosin heavy chain. To quantify contractile ring constriction kinetics, asymmetry and protein distribution, we created custom image-processing software. Depletion of endogenous Anillin, either in the absence or presence of GFP-tagged versions, disrupted ring asymmetry in several distinct ways. Normally, the contractile ring closes across the division plane unilaterally and is maximally asymmetric. After thorough Anillin depletion, asymmetry often completely fails; the ring centroid stays very close to the cell center. Quantitative analysis revealed an unexpected class, in which the ring closes asymmetrically until it approaches the spindle midzone. Then, asymmetry decreases and the ring closes near the center of the division plane. We are also using GFP-tagged Anillin truncations to understand how this multidomain protein amplifies contractile ring symmetry breaking. Preliminarily, Anillin’s myosin- and septin-binding domains seem necessary for asymmetry in cytokinesis, suggesting that crosslinking within the actomyosin cytoskeleton is important for this process.

638/B585
Spatial-Temporal Control of Cytokinesis in Fission Yeast: Positioning of Mid1/Anillin by Cdr2 Kinase and Nuclear Export and Activation of Myosin II Recruitment by Plo1 Kinase.
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Proper positioning of the cell division plane is required to maintain genome integrity and cellular function. In most eukaryotes, cytokinesis relies on a contractile actomyosin ring positioned by intrinsic spatial signals that are not well defined at the molecular level. Fission yeast cells assemble a medial contractile ring in response to positive spatial cues from the nucleus at the cell centre and negative spatial cues from the cell tips. These signals control the localization of the anillin-like protein Mid1, which defines the position of the division plane at the medial cortex where it recruits contractile ring components at the onset of mitosis. We have recently shown that Mid1 recruitment to the medial cortex is mediated by Cdr2 kinase which distribution is negatively regulated by Pom1 at cell tips (Almonacid et al.,CB 2009; Moseley et al.,Nature 2009; Martin and Berthelot-Grosjean,Nature 2009). In addition, Mid1 nuclear export links division plane position to nuclear position in early mitosis (Almonacid et al.,CB 2009). Cdr2- and nuclear export-dependent positioning of Mid1 constitute two overlapping mechanisms that relay cell polarity and nuclear positional information to ensure proper division plane specification. We now study how Mid1 gets activated at the onset of mitosis to recruit contractile ring components. We find that a region of 50 aa including Mid1 nuclear export sequence NES1 is required for Myosin II timely recruitment to medial cortical nodes at the onset of mitosis, even if Mid1 is forced out of the nucleus by additional mutations in Mid1 nuclear localization sequences (MidnsmΔ50-100 mutant). Mutations of 6 consensus phosphorylation sites for Plo1 kinase located between aa 1 to 100 produce a similar delay in Myosin II recruitment (Mid1nsm 6Ala mutant). We propose that Plo1 may activate Myosin II recruitment to medial cortical nodes by creating a binding site for Myo2 on Mid1 N-terminus at the onset of mitosis.

639/B586
PAR-4/LKB1 Regulates Actomyosin Contractility through Anillin during Early C. elegans Divisions.
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We are using the early embryo of C. elegans as a model to understand how PAR-4/LKB1, a conserved serine-threonine kinase, regulates cell polarity. Whereas the tumor suppressor function of PAR-4/LKB1 is well established, the mechanism by which it regulates polarity and asymmetric cell division, and its key downstream effectors during these events, are still unclear. We investigated the involvement of PAR-4 in the regulation of actomyosin contractility, a process
known to be required for proper cell polarization in the C. elegans embryo and other systems. Using time-lapse imaging of embryos expressing non-muscle myosin fused to GFP (NMY-2::GFP), we observed severe actomyosin contractility defects during the first cytokinesis of par-4 mutants. par-4 mutant embryos display multiple furrow ingressions and extensive membrane blebbing, resulting in a delay in the completion of cytokinesis. Furthermore, the cortical site of NMY-2::GFP recruitment at the presumptive cytokinetic furrow during anaphase was mislocalized towards the posterior of the embryo. To identify proteins acting with PAR-4 during cytokinesis, we carried out an RNAi-based, genome-wide screen for suppressors of par-4 embryonic lethality and identified ani-2, one of the C. elegans homologues of the actin-bundling protein Anillin. ANI-2 has high sequence homology with C. elegans ANI-1 and other metazoan Anillinrs in the domains thought to confer binding to MgcRacGAP/CYK-4. Rho and the septins, but is not predicted to bind actin or myosin, suggesting that it negatively regulates actomyosin organization and/or function. ANI-2 depletion by RNAi reverted all actomyosin defects observed in par-4 mutant embryos, suggesting that an increase of ANI-2 function is responsible for these contractility defects. Strikingly, immunolocalisation experiments showed that the loss of PAR-4 activity promotes ANI-2 recruitment to the cortex during cytokinesis. Our results thus support a model in which PAR-4/LKB1 inhibits ANI-2/Anillin accumulation at the plasma membrane, enabling proper actomyosin contractility and cytokinesis completion during early C. elegans embryonic divisions. Such reciprocal interaction may also control actomyosin contractility during embryo polarization.

640/B587
A Two-Step Mechanism for Transport of the Rho GTPase Flux Regulator MgcRacGAP to the Equatorial Cortex Before Cytokinesis.
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The formation of the contractile ring during cytokinesis is dependent on the small GTPase RhoA, which is activated in a precise zone at the cell equator. In a departure from traditional thinking about Rho activity regulation, whereby guanine nucleotide exchange factors (GEFs) initiate a particular event and GTPase activating proteins (GAPs) terminate that event, we have shown that the GAP activity of MgcRacGAP is necessary throughout cytokinesis for the formation and maintenance the Rho activity zone by promoting the rapid flux of Rho between the active, GTP-bound state and the inactive, GDP-bound state. The GTPase Flux mechanism helps cells maintain a focused Rho activity zone, which is necessary for forming a focused contractile ring and for successful cytokinesis. Here we show that MgcRacGAP is delivered to the equatorial cortex by a two-step mechanism. We examined MgcRacGAP localization in cells within the intact vertebrate epithelium using a gene replacement approach. MgcRacGAP puncta decorate microtubules near the equatorial cortex before ingresson, and these puncta congress and concentrate at the site where the contractile ring forms. Some microtubules decorated with MgcRacGAP puncta appear to make stable contacts with the equatorial cortex. Furthermore, MgcRacGAP puncta undergo directed movement along microtubules, especially near cell-cell boundaries. MgcRacGAP does not move along the entire length of astral microtubules, but instead first appears in a broad region around the equator and then focuses. Photobleaching demonstrates that MgcRacGAP puncta are dynamic, recovering quickly after photobleaching. Together, these results imply that MgcRacGAP is delivered to the equatorial cortex initially by diffusion from the spindle to a broad region surrounding the equator then focused by directed transport on microtubules. These results also represent, to the best of our knowledge, the first time that a gene replacement approach has been successfully employed to monitor cell division in a living, differentiated vertebrate epithelium within the context of the intact organism.

641/B588
Large-Scale Chemical Screen for Cytokinesis Inhibitors.
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Abnormalities in cytokinesis are often deleterious and can lead to aneuploidy and tumor formation in numerous cancer types. Understanding how cytokinesis is regulated is indispensable not only for gaining insight into this important biological process, but also for gaining information that be applied to cancer biology and genetic disorders, as well as to identifying new therapeutic drugs for these diseases. To address these objectives, we have designed a large-scale chemical screen using Dictyostelium discoideum. We have developed CYMPAQ - an analytical segmentation program used to identify both mitotic and cytokinesis inhibitors. We have screened over 22,000 compounds and have identified a number of inhibitors, which have been recapitulated in secondary screening. We are currently coupling these compounds with genetic screens to flush out cytokinesis regulators, as well as testing cross-reactivity in both mammalian cell lines and against organisms responsible for amebiasis.

642/B589
**Structural Studies of PRC1 (Protein Regulator of Cytokinesis).**
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Members of the evolutionarily conserved MAP-65 (Ase1, yeast; AtMAP-65, Arabidopsis; PRC1, human) family of proteins are microtubule associated proteins (MAPs) that crosslink microtubules in an anti-parallel orientation. Recent studies have shown that PRC1 is required for the formation of the central spindle, an anti-parallel array of microtubules required for completion of cell division. However, how orientation-specific crosslinking of microtubules is achieved by these non-motor MAPs is not known. Using cryo-electron microscopy and helical image analysis we have visualized the interactions of monomeric and tetrameric constructs of PRC1 on single microtubules and between two microtubules. Surprisingly the 3D maps of these constructs look similar. We have also used total internal reflection fluorescence (TIRF) microscopy to examine PRC1-microtubule interactions and microtubule crosslinking in real time. Combining the structural work with In Vitro fluorescence microscopy based assays have provided a better understanding of how PRC1 interacts with the microtubule lattice and how it forms bridges to neighboring microtubules.

643/B590
**MAP65-3 Mediates the Interaction between Kinesins and Microtubule Plus Ends during Cytokinesis in Arabidopsis.**
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During cytokinesis in plant cells, microtubules are organized in a bipolar phragmoplast array required for cell plate formation. Our previous work has shown that the Kinesin-12A B motors exclusively decorate the plus end of phragmoplast microtubules, and are required for the organization of anti-parallel microtubules in the phragmoplast in *Arabidopsis*. The MAP65/Ase1 family protein MAP65-3 cross-links microtubule plus ends in the phragmoplast. The loss of MAP65-3 leads to frequent failures in cytokinesis in *Arabidopsis*. In order to test whether MAP65-3 is required for the interaction between kinesins and phragmoplast microtubules, the *dyc283* mutant was used to determine how Kinesin-12A B and other phragmoplast-associated proteins behaved in the absence of MAP65-3. Our results showed that the localization of Kinesin-12A B at the plus end of phragmoplast microtubules was dependent on MAP65-3. Another phragmoplast specific kinesin, PAKRP2 became evenly localized along phragmoplast microtubules in the *dyc283* cells, instead of being concentrated in the phragmoplast midline as in the control cells. However, the localizations of the syntaxin KNOLLE and the dynamin DRP1C at the cell division site were not affected by the loss of MAP65-3. Our results suggest that MAP65-3 functions as a scaffolding factor for kinesins to interact with microtubule plus ends in the phragmoplast, but the protein is not required for vesicle transport along phragmoplast microtubules during cytokinesis.
Centralspindlin and SPD-1: Non-Redundant Interaction Partners Critical for the Central Spindle Formation during Cytokinesis.

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The central spindle and midbody are microtubule bundle structures that are critical for cytokinesis, providing sites for the accumulation of various factors essential for this process. Two evolutionarily conserved microtubule bundling factors, the centralspindlin kinesin/RhoGAP complex and PRC1 microtubule-associated protein, are essential for the formation of the central spindle and midbody. Although centralspindlin and PRC1 independently show microtubule bundling activity in vitro, it is poorly understood how both of them are required to form these microtubule bundle structures in vivo. Interestingly, a direct interaction between PRC1 and the RhoGAP subunit of centralspindlin has been reported. To gain insights into how this interaction might contribute to cooperative microtubule bundling, we have tested the evolutionary conservation of centralspindlin-PRC1 binding and found that it also occurs between C. elegans SPD-1/PRC1 and CYK-4/RhoGAP. Using yeast two-hybrid and In Vitro pull-down assays, we have narrowed down the binding domains on both SPD-1 and CYK-4. Furthermore, we have introduced several point mutations to the interacting regions and are now investigating the significance of the centralspindlin-SPD-1 interaction for central spindle/midbody organization using different biochemical and functional analyses.

**Neurotransmitters, Peptides and Receptors (645 – 669)**

645/B592

Antigen Presentation by Ventral Midbrain Dopaminergic Neurons: Implications in Parkinson's Disease.

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Parkinson's disease (PD) exhibits a progressive degeneration of dopaminergic neurons in the ventral midbrain (VM) but the cause of this neuronal loss is still poorly understood. Recent data suggest that neuroinflammatory mechanisms might contribute to the cascade of events leading to a chronic neuronal degeneration. The present experiments explore the immunological function of neuronal MHC class I antigens in VM cells. We show that MHC class I proteins were induced in postnatally-derived cultures murine VM neurons by treatment with recombinant IFN-γ. Moreover, CD8+ cytotoxic T lymphocytes that selectively recognize SIINFEKL peptide dramatically enhanced killing of MHC class I positive SIINFEKL treated neurons. We also examined the induction of the MHC-I in other brain regions (such as the cortex), but the expression of this antigen-presenting molecule was statistically lower than in ventral midbrain dopaminergic neurons. In addition, conditioned microglial medium obtained from microglial cultures treated with neuromelanin, lipopolysaccharide or alpha-synuclein induced MHC-I expression in VM neurons. These data reveal a novel inflammatory mechanism in neurodegenerative processes that could lead to the chronic neuronal death observed in PD.

646/B593

Prion Protein Transduces Signals after Binding to Laminin Gamma 1 Chain via Metabotropic Glutamate Receptors.

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The prion protein (PrPC) is a cell surface glycoprotein highly expressed in the nervous system, a conformationally modified isoform of which is associated with prion diseases. PrPC interacts with various proteins modulating neuronal survival, plasticity and memory. Transmembrane proteins are likely required for PrPC-mediated intracellular signaling with consequences for neuronal function/dysfunction. We show here that a laminin gamma-1 chain peptide induces PrPC-dependent neuritogenesis through an increase in intracellular Ca2+ and activation of PKC. Inhibition of phospholipase C, inositol 3-phosphate receptor and group I metabotropic glutamate receptors (mGluR1/5) abrogates both PrPC-dependent Ca2+ signaling and neuritogenesis. In addition, the expression of mGluR receptors in HEK 293 cells reconstitutes the signaling pathways mediated by PrPC-laminin gamma-1 chain peptide interaction. In agreement with a role of mGluR s in neuronal differentiation “in vivo”, treatment of neurons with mGluR1/5 agonist induces neuritogenesis. These data show that group I mGluRs are involved in neurotrophic properties of PrPC-laminin interaction.

647/B594
Reduced Degradation of Myelin Basic Protein by Anti-Sulfatide Antibody, TNF-α and IFN-γ in the Absence of Glia Maturation Factor.
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Multiple Sclerosis (MS) is an inflammatory demyelinating disease of CNS. The complex process of demyelination in MS is poorly understood. Glia Maturation Factor (GMF), discovered and characterized in our laboratory, is a highly conserved brain-specific protein. Recent findings indicate that GMF plays a critical role in regulating proinflammatory cytokines in the CNS and that GMF-deficient (GMF-KO) mouse failed to develop experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. In this study we show the effect of anti-sulfatide (RmAb) antibodies and inflammatory cytokines, TNF-α and IFN-γ in inducing myelin basic protein (MBP) degradation in myelin isolated from control wild type (WT) and GMF-KO mice. GMF was not detected in isolated myelin from WT and GMF-KO mice although it is present in brains of WT mice. Our results show that calcium-dependent neutral protease activity caused significantly elevated degradation of 18.5 and/or 17.5 kDa isoforms of MBP in WT myelin treated with RmAb or IFN-γ. In contrast, MBP degradation in isolated myelin from GMF-KO mice remained unaffected following treatment with RmAb, IFN-γ, or GM-CSF. Neither the 14 kDa isoform of MBP nor proteolipid protein (PLP) showed an elevated degradation compared to controls. A virtual absence of GM-CSF, TNF-α and IFN-γ in GMF-KO brain compared to WT was also evident when the animals were challenged with MOG 35-55. Taken together, our data suggests a role for GMF in the biochemical organization of myelin and thereby its effect on MBP degradation induced by RmAb and IFN-γ.

648/B595
Function of Rab5 Subfamily Members in Ngf-Induced Neuron Differentiation.
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NGF (nerve growth factor) signal transduction and membrane trafficking are key processes of neuronal development. Since Rab5 subfamily of small GTPases play important roles in regulation of early endosomal sorting and endocytosis of signaling receptors, we have investigated the requirement of each Rab5 subfamily member, including Rab5, Rab21, Rab22, and Rab31, in NGF-induced neurite outgrowth in PC12 cells. Wild-type (WT), dominant active (DA), and dominant negative (DN) mutants of each Rab was expressed in PC12 cells via the pBI/eGFP vector, which expressed eGFP (enhanced green fluorescent protein) simultaneously to identify the transfected cells by fluorescence microscopy. In the case of Rab22, shRNA was used to knock-down endogenous Rab22 due to low expression of its DN mutant. The effects on NGF-induced neurite outgrowth were determined and compared. Our results showed that the DN mutants of Rab5 and Rab21 dramatically increased neurite outgrowth in PC12 cells; while their WT and DA counterparts inhibited this differentiation process. Expression of Rab31, in any form,
showed no effects on the neurite outgrowth. In contrast, Rab22 knock-down by shRNA significantly inhibited neurite outgrowth, while Rab22 WT or DA mutant showed little effects. The data suggest that Rab22-mediated early endosomal sorting and membrane recycling is important for NGF-induced neurite outgrowth. Furthermore, we found that Rabex-5 functions as a Rab22 effector in promoting neurite outgrowth. Overexpression of Rabex-5 or Rabex-5:D314A, a mutant defective in the Rab5 GEF activity, significantly enhanced neurite outgrowth, suggesting that the Rab22-Rabex-5 signaling cascade in neurite outgrowth is independent of Rab5 activation.

649/B596
Alterations of EHD1/EHD4 Levels Disrupt Axonal Targeting of L1/NgCAM.
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Polarized targeting is crucial for maintenance of neuronal polarity. The molecular mechanisms on how membrane proteins are selectively enriched in specific compartments in neurons remain unknown. We demonstrated previously that L1/NgCAM travels to the axon on an indirect transcytotic route via the somatodendritic plasma membrane and subsequent sorting to the axon in somatodendritic endosomes. Here, we further characterize the role of endosomal-related protein, EHD1/RME-1, in regulating L1/NgCAM recycling in neurons. EHD1/Rme-1 is a member of the Eps15 homology domain (EHD) family that also consists of other three highly homologous paralogs, i.e. EHD2, 3 and 4 in mammalian cells. EHD1 has been reported to regulate the recycling of AMPAR and transferrin from endosome to plasma membrane. We show here that overexpression of EHD1 leads to impairment of L1/NgCAM endocytosis in neurons and reduces the polarization of NgCAM in axon. In contrast, the expression of EHD1 does not affect the internalization of transferrin receptor. Interestingly, the inhibition of NgCAM endocytosis by EHD1 is not observed in fibroblasts. This indicates that endocytosis defect caused by the overexpression of EHD1 is cell type and cargo specific. Intriguingly, overexpression of EHD4, but not of EHD3, also causes a defect in NgCAM endocytosis and leads to reduced axonal polarization of L1/NgCAM. EHD family of proteins contains a central helical region that mediates self-oligomerization and hetero-oligomerization within the members of the family. In fact, an endogenous complex of EHD1 and EHD4 has been previously described. We find that a mutant of EHD1 that is impaired for oligomerization does not inhibit L1/NgCAM endocytosis. Strikingly, simultaneous expression of EHD1 and EHD4 rescues the NgCAM endocytosis defect whereas simultaneous expression of EHD4 and the oligomerization-impaired EHD1 does not rescue NgCAM endocytosis. Our observations suggest a model in which the relative levels of EHD1 to EHD4 are important for NgCAM endocytosis in neurons. We propose that L1/NgCAM uses a specialized endocytosis pathway in neurons dependent on EHD1 and EHD4, which may act as hetero-oligomeric complexes in this pathway.

650/B597
Functional Selectivity in Serotonin 2A (5-HT2A) Receptor Trafficking.
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The serotonin receptor 2A (5-HT2A) is the target of several drugs, including ones for the treatment of psychiatric disorders and also recreational hallucinogenic drugs. Signal transduction cascades modulated by these ligands are poorly understood. The 5-HT2A is also a useful model system to study functional selectivity in GPCRs. Functional selectivity refers to ligand-dependent selectivity for signaling cascades at a receptor - the degree to which a pathway is modulated depends on the ligand, challenging definitions of ‘agonist’ and ‘antagonist.’ We describe the effects of several ligands (agonists as well as antagonists) on signaling and trafficking of the rat-5-HT2AR-EGFP expressed in HEK293 cells. We have earlier shown that agonists serotonin (5-HT) and dopamine bring about receptor internalisation and recycling. Interestingly, so do several other ligands including antagonists such as some antipsychotic drugs. We show that different biochemical pathways are involved in receptor internalization mediated by various ligands. Thus, agonists DOI and dopamine and some antagonists (including some antipsychotic drugs) bring
about rat-5-HT_{2A}-EGFP internalization and do not require PKC activation. We show that by abrogating a putative PKC-phosphorylation site on the receptor, we selectively inhibit internalization by ligands that require PKC-activation, leaving others unaffected. Also, we show that levels of receptor internalization vary significantly with the ligand used. Post internalization, the rat-5-HT_{2A}-EGFP recycles to the cell surface in varying times, depending on the ligand used. We have previously described that in HEK293 cells, 5-HT and dopamine bring about receptor recycling in 2.5 hours. We now show that agonist DOI and antagonists bring about recycling in 7.5 hours. Finally, it appears that receptor recycling requires de-phosphorylation, as protein phosphatase 2A (PP2A) activity is required for receptor recycling brought about by ligands that require PKC-activation for receptor internalization while not required for recycling effected by other ligands. These differences in internalization and recycling highlight a novel form of functional selectivity in GPCRs.

651/B598

Nociceptin and Nociceptin Derivatives Are Chemorepellents in Tetrahymena thermophila.

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Nociceptin is a cationic neuropeptide which has been implicated in myriad biological functions, including pain, cognition, memory, and addiction pathways. Since the nociceptin receptor was discovered over a decade ago, much has been learned about the physiology of this peptide. The mechanisms of nociceptin signaling appear to be diverse. In hippocampal tissue, pharmacological inhibition of ERK suppresses signaling through the nociceptin receptor (Goeldner et al., 2008). Nociceptin receptors appear to directly interact with, and inhibit, N-type calcium channels (Altier and Zamponi, 2008). Nociceptin signals activate neuronal nitric oxide synthase (nNOS) (Xu et al., 2007), and inhibit cAMP-induced gene transcription (Gottlieb et al., 2006). Tetrahymena thermophila, as a model system in which to study nociceptin pathways. In Tetrahymena, nociceptin, as well as the nociceptin derivatives nociceptin-NH_{2} and nociceptin-Arg_{14}Lys_{15} all serve as chemorepellents in our behavioral assay. All three compounds had EC_{100} values in the micromolar range in our behavioral assay. The chemorepellent activity of nociceptin was blocked by the addition of genistein (100 μg/ml), suggesting tyrosine kinase involvement in the avoidance response. Avoidance to nociceptin was unaffected by the addition of diadzein (100 μg/ml), GDP-μ-S (1 mM), Rp-cAMPs (50 μM), neomycin sulfate (5 μM), calphostin C (10 μM), or U-73122(1 μM), suggesting that G-proteins, cAMP, and phospholipaseC/PKC signaling are not involved in the nociceptin response in this organism. Further experimentation will reveal more information about the nature of the proposed tyrosine kinase cascade, and how this activity relates to the ciliary reversal, ultimately leading to avoidance behavior in Tetrahymena.

652/B599

Myostatin Expresses in Neurons Related to Olfactory System in the Brain.

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Myostatin, which is a negative regulator of skeletal muscle growth, belongs to the transforming growth factor-β (TGF-β) superfamily. Recently, it has been reported that myostatin mRNA is expressed in different brain regions of different vertebrates. However, the details of myostatin localization and function in the brain remain to be investigated. Therefore, we tried to examine the intracellular expression of myostatin and activin receptor type II (ActRIIB), which is the receptor of both myostatin and activin in mouse brain, and to further reveal the influence on neural activation of myostatin by the intracerebroventricular injection. Expression of myostatin, ActRIIB and activin a was analyzed by RT-PCR, in situ hybridization and immunohistochemistry using Male C57BL/6 mice aged 14 weeks-old. An intracellular existence of myostatin was identified by the immunostainings using mABs of myostatin and its propeptide. Myostatin was restricted in the mitral cells of olfactory bulb and in the neurons of olfactory cortex. On the other hand, activin A, which is a member of TGF-β superfamily, localized in neural cells of the hippocampus CA1 region, paraventricular thalamic nucleus and solitary nucleus. ActRIIB was observed in the cells of olfactory bulb, olfactory cortex, the hippocampus CA1 region and paraventricular thalamic
nucleus except for solitary nucleus. The findings show that myostatin and activin are differentially localized in mouse brain. When myostatin or activin was injected in the lateral ventricle, c-Fos, an activation marker for neural cells, was detected in neuronal cells of olfactory bulb of myostatin-injected mice but not activin-injected mice. The present study first describes that myostatin is expressed in the mitral cells of olfactory bulb and affects the olfactory system by autocrine and paracrine pathway.

653/B600
Synergy between Purinergic and Cholinergic Signalling in Rabbit Lacrimal Gland.
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Introduction. We have previously shown that simultaneous stimulation of the muscarinic M3 receptor and the purinergic A1 receptor results in a secretion from lacrimal cells larger than a purely additive response, indicating a synergy between the signalling pathways (Edman et al., Exp Eye Res:86:110, 2008). More recently, our results also indicates the presence of both the purinergic A2a and A2b receptors (Gierow et al, Invest. Ophthalmol. Vis. Sci. 49 (ARVO abstract) p. 2449, 2008). Therefore, it was of interest to investigate if a similar synergy could be observed between the A2 receptors and the cholinergic receptor. Methods. Rabbit lacrimal gland acinar cells were prepared according to our standard procedure (Gierow et al., Am. J. Physiol. (Cell Physiology) 271: C1685, 1996; Andersson et al, Glycobiol. 15: 211, 2005) yielding single cells that were placed in a serum-free culture medium on standard culture plates coated with Matrigel for two days. The cultured cells were rinsed briefly and secretion was measured after a pre-incubation for 30 min at 37 C, with buffer alone, followed by incubation for an additional 30 min w./w.o. secretagogues and/or inhibitors as indicated. Basal secretion and regulated secretion was determined enzymatically as secreted beta-hexosaminidase activity. Results. A strong synergistic effect was observed when carbachol, a cholinergic agonist, and CPCA, an A2-agonist, were combined. The synergy was observed already after 5 min of incubation and at a low concentration of CPCA (0.1 μM). A response more than two-fold higher than expected was observed after 30 min of incubation at 10 μM CPCA. The synergistic effect was significantly reduced to levels comparable with Cch alone by the A2b antagonist PSB 1115, but not by SCH 52261, an A2a antagonist. Conclusions. Our results indicate that also the A2b receptor can potentiate the response to carbachol. The mechanism of potentiation remains unclear but preliminary studies indicate that intracellular Ca2+ is involved in the process. Support. Supported by Crown Princess Margareta’s Eye Research Foundation (JPG) and University of Kalmar Faculty Research Grant (DD & JPG).

654/B601
Characterization of Behavioral Defects in PAM-1 Mutants in Caenorhabditis elegans.
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<p>Puromycin-sensitive aminopeptidases (PSA) are a widely conserved family of metalloproteases that hydrolyze N-terminal amino acids. The PSA homolog in Caenorhabditis elegans, PAM-1, has been localized to neurons, intestinal cells, and embryos (Brooks et al). While it has been implicated in meiotic exit and anterior-posterior axis specification in the early embryo (Lyczak et al), its role in the nervous system remains largely unstudied. Recent findings have shown that human PSA protects against tau-induced neurodegeneration In Vivo and directly proteolyzes tau In Vitro (Karsten et al). Such findings underscore the need for a more comprehensive understanding of the role of PSA in the nervous system. The objective of this study is to elucidate the possible functions of PSA in neurons by characterizing the behavioral defects of PAM-1 in C. elegans. Preliminary results indicate that motor activity, osmosensitivity, and mechanosensitivity are significantly impaired in pam-1 mutants. The or282 (early stop codon) and or403 (late stop codon) pam-1 alleles exhibit 72% and 24% decreases in motor activity and 60% and 78% decreases in osmosensitivity, respectively, versus the wildtype. Mechanosensitivity is also impaired in pam-1 mutants with the or282 allele, which display a 48% decrease in nose

655/B602

Comprehensive Labelling of Melanopsin Expressing Retinal Ganglion Cells and Mapping Their Central Projection in Mouse.

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Melanopsin is an opsin class of photopigment exclusively expressed in a small subset of retinal ganglion cells (mRGCs) that are intrinsically photosensitive. These mRGCs project their axons to the suprachiasmatic nucleus (SCN) and a few other brain regions that directly or indirectly regulate non-image forming visual processes including circadian photoentrainment, pupil constriction, pineal melatonin regulation and light regulation of activity/rest. Identifying the full complement of mRGCs and their central projection is critical to understanding the cellular basis of melanopsin function. However, the existing methods to mark melanopsin cells and map their projections are insufficient. Therefore, we have generated transgenic reporter lines to specifically label the mRGCs and comprehensively study the projections of mRGCs in adult mice. We generated a mouse with targeted insertion of Cre-recombinase into the native melanopsin locus and bred it with a Z/EG or Z/AP mice. This strategy allows Cre-dependent expression of green fluorescent protein (GFP) or human placental alkaline phosphatase (AP) from a strong β-actin promoter. In the resultant mice melanopsin cells are marked with a GFP or an AP marker which can be visualized by fluorescence or histochemical staining respectively. In the retina of Opn4Cre/+;Z/EG mice, GFP expressing cells were mostly found in the retinal ganglion cell (RGC) sub-layer, and these cells had extensive dendritic arborization characteristic of the mRGCs. An average of 131 GFP expressing cells/mm² (±25.4, SD, n = 3) were found in these retina, 42.6% of which also expressed immunologically detectable levels of melanopsin. In Opn4Cre/+;Z/AP mice, the mRGCs strongly innervate the SCN. Additionally, mRGCs axon termini also sparsely innervate various other hypothalamic regions. Surprisingly, the AP staining also revealed extensive projection of the mRGCs in the lateral geniculate complex which is involved in image-forming vision. This implies mRGCs may play some role in patterned vision. In summary, we found that the projection patterns of mRGCs were much more extensive than previously reported.

656/B603

Lysophosphatidic Acid (LPA) Is a Chemoattractant and a Mitogen in Tetrahymena: Separation of These Two Response Pathways by a GPCR Knockout Mutant.

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Tetrahymena are free-swimming eukaryotic unicells that change their swimming behavior in response to many types of stimuli to direct them away from dangerous areas and towards preferred areas, such as food sources. Some of the cellular receptors and pathways involved may be sufficiently conserved to allow the use of Tetrahymena as a simple model system for chemosensory transduction studies in these “free swimming sensory cells”. In general, chemoattractants cause somatic hyperpolarization, faster forward swimming and more linear swim paths while chemorepellents cause depolarizations, slower forward swim speeds and action potentials at threshold concentrations to cause bouts of backward swimming. Since many chemosensory receptors are GPCRs (G-protein coupled receptors) we identified several possible GPCR sequences in the Tetrahymena Genome Database and used them to generate knockout mutants by homologous recombination. One mutant, G6, is a complete macronuclear knockout of GPCR6 (THERM_00925490). This mutant is unresponsive to the chemoattractants lysophosphatidic acid (LPA,10μM) and proteose peptone (0.1%), suggesting that gpcr6 may be a chemosensory receptor. Since the LPA and proteose peptone responses cross-adapt in wild type and both chemoattractant responses are missing G6, it is possible that both stimuli share a common receptor. The G6 mutant is also more resistant to Ba++ paralysis than wild type,
suggesting that GPCR6 may have some constitutive effects on divalent ion permeabilities as well. The G6 mutant can be phenocopied by either pertussis toxin or calphostin C, suggesting a G-protein and protein kinase C involvement in this chemoresponse pathway. LPA is also a potent mitogen, causing a 2-3 fold increase in cell number when added to growing cultures in micromolar amounts. Since LPA is also a mitogen in the G6 mutant, the LPA chemotraction and mitogen pathways may be different in these cells and each may have different receptors. Supported by NSF grant MCB-0445362 to T.M.H.

657/B604

**Neuferricin, a Novel Extracellular Heme-Binding Protein, Promotes Neurogenesis by Inhibiting Cell Proliferation.**

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We identified a novel extracellular heme-binding protein and named it neufericin. The recombinant mouse neufericin produced in cultured cells was secreted efficiently into the culture medium. Mouse neufericin mRNA was expressed exclusively in the brain at the embryo stage and gradually increased during development. In contrast, it was widely expressed in postnatal tissues including the brain, heart, adrenal gland, and kidney. Mouse neufericin has 263 amino acids. It has a cytochrome b5-like heme/steroid-binding domain and appeared to bind hemin because neufericin solution, but not a solution of neufericinΔHBD (a mutant lacking the heme-binding domain), was tinged with brown and had an absorbance peak at 402 nm. In addition, the experiment with anti-neufericin antibody proved that the endogenous neufericin detected in the culture medium of Neuro2a cells using heme-affinity chromatography was associated with hemin. Inhibition of endogenous neufericin by RNA interference excessively promoted proliferation and suppressed neurite outgrowth during the induction of differentiation in Neuro2a cells. Addition of recombinant mouse neufericin, but not neufericinΔHBD, suppressed proliferation in Neuro2a cells and rescued from the effects of neufericin RNAi. In primary cultured mouse neural precursor cells, recombinant mouse neufericin exhibited the ability to promote neurogenesis. The identification of neufericin, a novel extracellular heme-binding protein with cytochrome b5-like heme/steroid-binding domain and its neurogenic activity, provide new insights not only into brain development but also the function of heme-binding proteins as extracellular signal transmitters.

658/B605

**Existence of Secretory Granule (Large Dense Core Vesicle)-Based Inositol 1,4,5-Trisphosphate-Dependent Ca2+ Signaling System in Astrocytes.**

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Astrocytes are now known to secrete a number of signaling molecules that participate in the cell-to-cell communication, involving both the neurons and the glial cells. There exist generally two types of secretory vesicles in astrocytes, one being the translucent small synaptic-like vesicles and the other the large dense-core vesicles. To clarify the identity of the large dense core vesicles and to elucidate the IP3-dependent Ca2+ signaling mechanism in astrocytes, we investigated the presence of typical secretory granule marker proteins, chromogranin B (CGB) and secretogranin II (SgIIL), in astrocytes. We have also examined the potential presence of the IP3Rs in the large dense core vesicles of astrocytes. In the present study, we found the localization of two typical secretory granule marker proteins chromogranin B and secretogranin II in the large dense-core vesicle of astrocytes, thereby identifying the large dense-core vesicles as secretory granules. We also found the presence of large amounts of all three isoforms of the IP3Rs in secretory granules of astrocytes. Hence, in view of the roles of secretory granules in secretory cells as the major IP3-sensitive intracellular Ca2+ store, the existence of secretory granules in glial astrocytes strongly suggests the existence and operation of an IP3-sensitive intracellular Ca2+ store of astrocytes in the form of secretory granules (large dense-core vesicles).
Function of Semaphorin 6D on Cerebellar Granular Precursors and Medulloblastoma Cells.

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During development, nervous progenitors are responsive to microenvironment. Precisely, under guidance cues progenitors migrate on long distances to their specific targets where they fully differentiate. During tumoral process, cell migration is often impaired leading to invasive growth. Semaphorins, first described as guidance cues for axons have been recently involved in cell migration, apoptosis, nerve regeneration and tumor progression. We focused on semaphorin 6D (Sema6D), first described in the collapse of DRG neurons and migration of embryonic cardiac cells (Qu et al., J. Biol. Chem. 2002; Toyofuku et al., Genes Dev. 2004). Our objective is to explore the role of Sema6D on cerebellar granule cell precursors (GCPs) compared to Medulloblastoma cell lines DEV and DAOY. We demonstrated that Sema6D is located in the external granular layer during cerebellum development and induced apoptosis in the granule cells (GC) expressing only Sema6D. Using a TUNEL and caspase 3 active assays, our results showed a 30% increase of GCPs apoptosis (92 ± 2.7) when seeded with GCPs expressing Sema6D versus (31 ± 1) with control cells (n=4; p<0.001). Furthermore, Sema6D acts as a repulsive guidance cue for GC axons. Medulloblastoma cell lines, Dev and Daoy, which expressed only plexinA1, did not undergo apoptosis but an increase of migration by 30% under Sema6D. Using a chemiotaxis assay, DEV cells migration is significantly increased when they are seeded with Sema6D (22.3 ± 2.3) compared without Sema6D (12 ± 0.7; p<0.01). The overexpression of mutated form of plexin A1 demonstrated the importance of plexinA1 in the migration of medulloblastoma cells. on medulloblastoma biopsies a large expression of plexinA1 is detected pointing out a role of Sema6D in the progression of Medulloblastoma. Conclusion : GCPs expressed Sem6D while Medulloblastoma cells expressed only the receptor PlexinA1. Apoptosis is observed under GCPs exposure to Sema6D while in Medulloblastoma cells ,migration is observed depending of plexinA1 under Sema6D

Chemical Transmission between Dorsal Root Ganglion Somata through Schwann Cell Intervention.

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Sensory neurons relay action potentials directly from the periphery to the spinal cord along a continuous axon that does not pass through the cell body located off a side branch in the dorsal root ganglion (DRG). Indeed, it is generally supposed that the neuronal somata are electrically independent. However, action potential backfiring from non-stimulated neurons has been reported and suggests at least some form of inter-neuron transmission. It has also been reported that DRG neurons activate their enveloping Schwann cells via purinergic transmission (Zhang et al., 2007 Proc Natl Acad Sci). Objective: to test if neighbouring DRG neuron somata can communicate via a di-synaptic mechanism through an intervening Schwann cell. Methods: Chick DRG neurons were gently dissociated to retain small clusters with their enveloping Schwann cells. The patch clamp technique was used to record ion currents from single cells or two cells simultaneously and variety of pharmacological agents were used to dissect the chemical signalling pathways. Results: Morphological studies confirmed that DRG neurons are separated by one or two glial cell layers. Direct recording from DRG-associated Schwann cells detected intermittent current transients that were identified as miniature excitatory spontaneous currents (mEPGs; n=5). Recordings from DRG neurons held at -80 mV detected variable membrane current noise current (n= 11) that was blocked by the nicotinic antagonist, d-tubocurarine (n=5) and that was enhanced by ATP application onto its associated Schwann cell. Depolarization of one DRG neuron in paired recordings triggered increased ACh noise 5 to 40 seconds later in one or both cells (n=9). Conclusion: Our results suggest a form of chemical transmission whereby a
DRG soma releases ATP to excite its enveloping Schwann cell which in turn secretes acetylcholine to activate neighbouring DRG somata. This ‘sandwich synapse’ represents a unique mechanism of inter somatal communication.

661/B608
Structure of Botulinum Neurotoxin Serotype B in a Lipid Bilayer at Multiple pH Levels.
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Botulinum Neurotoxin (BoNT) is the most deadly bacterial neurotoxin known. Secreted by the bacteria *Clostridium botulinum*, it has seven serotypes (A-G). BoNT B is a Zn2+ -endoprotease that acts on the exocytotic machinery at the neuromuscular synapse by cleaving unique components of the SNARE complex thus inhibiting synaptic vesicle fusion and subsequent neurotransmitter release. BoNTs are produced as 150 kDa single polypeptide chains, which are cleaved into 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked by a disulfide bond. The 100 kDa HC contains a 50 kDa translocation domain and a 50 kDa receptor binding domain. The 50 kDa LC is made up of a single catalytic domain. The pivotal event in the BoNT intoxication is the translocation of the LC across the cell membrane of the endocytosed vesicle into the cytosol. It has been postulated that upon a reduction in pH (7-4), BoNT undergoes a conformational change whereby the translocation domain inserts into the membrane forming some type of a channel through which the LC can be transported. Previous electron cryomicroscopy studies have shown the potential presence of a membrane channel, however, the resolution of these structures was at the outer limit of being able to detect a channel structure. Here, we have crystallized BoNT B at pH 6.0, 5.6, and 4.8 in the presence of lipid (DOPS) and ganglioside (GD1a). These structures show no presence of a membrane-spanning channel. These results lead us to believe that the presence of a protein channel may not be necessary for the translocation of the BoNT LC and that possibly the unfolding or conformational change of the LC itself disrupts the membrane enough to allow it to pass through.

662/B609
Catharanthine Alkaloids Are Noncompetitive Inhibitors of Muscle-Type Nicotinic Acetylcholine Receptors.
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This is an attempt to characterize the binding sites and the mechanisms of action of several catharanthine alkaloids including ibogaine, vincristine, and vinblastine, on muscle-type nicotinic acetylcholine receptors (AChRs), by comparing their pharmacological properties with that for the well characterized high-affinity noncompetitive antagonist (NCA) phencyclidine (PCP). In this regard, structural and functional approaches were used including radioligand equilibrium and competition binding assays using [3H]ibogaine and the analog of PCP, [piperidyl-3, 4-3H(N)]-N-(1-(2 thienyl)cyclohexyl)-3,4-piperidine ([3H]TCP), Ca2+ influx determinations, thermodynamic and kinetic measurements using column-immobilized Torpedo AChRs, and molecular docking and dynamics studies. The results established that: (a) the alkaloids inhibit (±)-epibatidine-induced Ca2+ influx in embryonic muscle AChRs with the following potencies (in μM): ibogaine > vinblastine > vincristine, that are slightly higher than that for PCP, (b) the alkaloids inhibit [3H]TCP binding, and ibogaine and PCP inhibit [3H]ibogaine binding, to the desensitized Torpedo AChR with higher affinity compared to the resting AChR, (c) ibogaine binds to the Torpedo AChR by an enthalpy-driven process, and (d) ibogaine interacts with a binding domain located between the serine (position 6') and valine (position 13') rings, by a network of van der Waals and polar interactions. Collectively our data indicate that catharanthine alkaloids block agonist-activated ion channels by interacting with a binding domain that is shared with PCP located between the serine and valine rings. This supports the view that the catharanthine moiety, which is shared by ibogaine and vinca alkaloids, is a minimum structural requirement for the interaction of these
molecules with the ion channel. In addition, ibogaine and vinca alkaloids may induce and maintain the desensitized state by a mechanism where their dissociation rates are decreased.

663/B610
Glutamate Induces a Specific Nuclear Size Change in Astrocyte through Aquaporin 1.
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Glutamate (Glu), an evolutionary selected excitatory messenger between neurons and astrocytes, was found to specifically induce nuclear swelling in astrocytes, but not in any other cell types. This was a direct and specific effect of Glu uptake via transporters, and not receptors, metabolites, ion or osmotic changes. Interestingly, Glu-induced nuclear swelling was found to require aquaporin 1 (AQP1), which was uniquely expressed and located on the nuclear membrane of astrocytes. Moreover, over-expression of AQP1 in astrocytes accelerated and enhanced nuclear swelling and if inhibited would prevent swelling. Our study discovers a specific nuclear response in astrocytes to exogenous Glu. This new mechanism might be an unknown specialization in astrocyte to facilitate the communication of excitatory information between neuron and astrocyte.

664/B611
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Recent several evidence has demonstrated that neuropeptide, in particular substance P(SP), plays an important role in various wound healing. The inflammatory response is playing an important role in the acute and chronic phases of spinal cord injury(SCI). We explored the effect of exogenous SP on functional recovery after SCI. SP was intravenously injected three times, immediately, 24h and 48h after contusive SCI. RT-PCR analyses revealed that SP injection increased expression of IL-10 mRNA, an anti-inflammatory cytokine but decreases TNF-α, IL-6, iNOS expression, potent pro-inflammation cytokines. at five days after SCI, the immunohistochemical analysis showed that SP treatment dramatically reduced ED1 positive cells in the lesion site when compared to the vehicle control. In addition, SP treatment significantly reduced the number of TUNEL positive cells as compared to that of the vehicle control and Basso-Beattie-Bresnahan(BBB) locomotor open field behavioral scores were significantly higher in SP-treated rats than vehicle-treated rats. These data support that SP treatment modulated inflammatory responses in the SCI, resulted in lower cell death, and improved functional recovery after SCI, possibly by stimulating anti-inflammatory response and mitigate the injury-mediated cell death.

665/B612
Role of the G-Protein Coupled Receptor, SER-7, in Adaptation and withdrawal Responses to Chronic Serotonin Exposure in C. elegans.
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We are investigating the behavioral responses and neural pathways modulated by chronic elevations of serotonin (5-HT) levels using the roundworm, C. elegans. In C. elegans long-term exposure to 5-HT leads to a diminished response over time, a phenomenon referred to as behavioral adaptation. A subsequent rebound effect occurs upon removal of worms from 5-HT, which is referred to as withdrawal. Both of these responses associated with chronic 5-HT exposure can be quantified by examination of the worm's egg-laying behavior. Acute exposure to 5-HT normally increases egg-laying rate two to three fold in wild-type worms, while longer overnight exposure results in a return of egg-laying rates to untreated control levels. Removal from 5-HT results in a further decrease in egg-laying rates to about 20% of untreated control levels. We initiated a study using a candidate gene approach to identify proteins mediating...
adaptation and withdrawal responses. We identified SER-7, a 5-HT-dependent G-protein coupled receptor (GPCR), is important for mediating the withdrawal responses. SER-7 has previously been shown to couple to Gαs and mediate the stimulatory effects of 5-HT on egg laying along with another GPCR, SER-1. ser-7 mutant worms show a two-fold increase in egg-laying rate during withdrawal rather than a decreased egg-laying rate seen in wild-type worms. A quadruple mutant lacking all of the identified 5-HT receptors in C. elegans, except SER-7 was tested and displayed normal withdrawal responses further demonstrating a role for SER-7 in the withdrawal response. Double mutants lacking both stimulatory receptors, SER-7 and SER-1, are also deficient in withdrawal while ser-1 mutants alone show normal withdrawal responses. This result suggests that SER-7 acts downstream of the SER-1 pathway. Our results suggest a pathway for the withdrawal response involving SER-7 presumably through activation of a cAMP-dependent pathway.

666/B613
A1 and A20 Expression in Cortex, Hippocampus and Brainstem Induced by LPS and Their Relationship with Apoptosis in a Shock Endotoxic Model.
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Endotoxic shock it’s a consequence of a poor regulated immune response when organisms are exposed to gram-negative bacteria and these release lipoposachryde (LPS). In this way LPS cause damage trough a badly immune response, release inflammatory substances like TNF-α, IL-1, IL-4 e IL-6. This event active regulatory signals of apoptosis induced by TNF-α, A1 and A20 proteins. Our aim was to study the A1 and A20 expression in Sprague-Dowley rat brain and the relationship between endotoxic shock-apoptosis in cortex, stem and hippocampus. We observed morphological changes in tissues related with apoptosis and necrosis using HE stain. With TUNEL, we can verify the cell death in different exposition times to LPS; this phenomenon can be observed in tissues with 30 minutes after exposition to LPS. The western blot assay shows the protein expression with evident changes in different exposition times. This pattern of antiapoptotic proteins expression was clear after 30 minutes after exposition to LPS. A1 and A20 proteins are expressed in endothelial cells in a proinflamatory response to protect it versus apoptosis mediated by TNF and ceramides. A1 and A20 are members of Bcl-2 proteins and blocks the transcriptional process mediated by NFkB. This investigation shows the rol of NF-kB in immune system with A1 and A20 expression.

667/B614
A Novel Protein That Potentiates the Function of Mammalian Odorant Receptors.
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Mammalian olfaction begins with the activation of odorant receptors (ORs), which are class a G-protein coupled receptors (GPCRs) expressed on the surface of olfactory sensory neurons (OSN), by volatile odorant molecules. While a large number of ORs have been identified, little is known about the complex mechanism behind OR ligand selectivity and combinatorial coding. Previous studies in our laboratory have demonstrated that OR expression in heterologous cells can be enhanced by a group of transmembrane proteins RTP1 and RTP2. Since OR activity remains lower in heterologous cells in comparison to that in the sensory neurons, we hypothesized that other proteins may form functional interactions with ORs that are required for OR activation or trafficking. To screen for accessory proteins that enhance the activity of ORs, we used a luciferase assay system that quantities OR activity upon odorant stimulation. Here, we report the discovery and characterization of an OR-Enhancing Protein 3 (OREP3) as a membrane receptor that potentiates the activation of a number of ORs when it was transiently expressed in HEK293T and Hana3A cells. Coexpression of OREP3 lowered the EC50 by a factor of 10 and increased the maximum luciferase response upon OR stimulation by 30-50%. Unlike RTP1 and RTP2, OREP3 did not seem to enhance cell-surface expression of the ORs, suggesting that the action of OREP3 is distinct from known accessory factors. Furthermore, the ability of OREP3 to enhance OR activation appeared to be significantly enhanced in the presence of RTP1, which indicated that the effect of OREP3 is synergistic. We have found that OREP3...
forms stable complexes with ORs when they are coexpressed in HEK293T cells. These results suggested that POREP3 and ORs may form heteromeric, functional interactions that leads to the potentiation of OR activation by POREP3. We have used POREP3 to develop a novel screening system that increased the efficacy of our screening system for the ligands of orphan ORs. This information will be integral to deciphering the molecular mechanism of olfaction in mammals.

668/B615
Short-Chain Fatty Acids Regulate the Sympathetic Nervous System via the GPR41.
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Short chain fatty acids (SCFAs), including acetate, propionate and butyrate, are primary derived from bacterial fermentation of carbohydrates in the colon. They not only contribute significantly to caloric intake but also function as signaling molecules. GPR41 was identified as the G protein-coupled receptor for SCFAs. Recently, it was reported that regulation of colonic motility in small intestine and leptin production in adipocytes was induced by SCFAs through the GPR41. However, the physiological functions of GPR41 are little known, because GPR41 expression was low and species difference in these tissue. Here we report that mouse GPR41 mRNA is expressed most abundantly in sympathetic ganglion than other tissues during from developmental stage to adult stage. Therefore, we generated GPR41 knockout mice to elucidate the physiological roles of GPR41 in sympathetic nervous system. During developmental stage, the mildly differentiational retardation of sympathetic ganglion in GPR41-/- mice was observed. In adult stage, Deletion of the GPR41 led to a decrease of sympathetic activity with heart rate, blood pressure and an increase of noradrenaline concentration in heart and sympathetic ganglion but not catecholamine in adrenal gland. The heart rate had not been significantly different by the treatment with propranolol as beta-adrenoceptor blocker. In addition, in primary cultured mouse sympathetic neurons, the treatment with SCFAs promoted the release of noradrenaline and neurite extension. The sympathetic neurons from GPR41-/- mice did not exhibit these effects. Therefore, GPR41 may regulate the sympathetic nervous system by modulating the release of noradrenaline in sympathetic neuron. These results will provide new insights into SCFAs as a neurotransmission regulator and neurotrophic factor through the GPR41.

669/B616
Maternal and Postweaning Diet Interaction Alters Hypothalamic Gene Expression and Response to a High-Fat Diet in Male Offspring.
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We tested the hypothesis that consumption of excess saturated fats during pregnancy and lactation contributes to adult metabolic dysfunction and that these disturbances can be further influenced by the postweaning diet. Adult male offspring from chow-fed dams were compared with males from dams fed a diet high in saturated fat (45 kcal/100 kcal) before mating, during pregnancy and lactation. Offspring were weaned to a standard chow diet or high fat diet. Animals were killed at 120 days after a 24-h fast. Although there was no difference in birth weight between offspring from dams fed control food compared to those fed a high-fat diet, wean weight was significantly higher in offspring from the high-fat fed dams. By day 120 of the postnatal period, weight gain and energy intake were significantly increased in the fat-fed offspring from fat-fed dams (P <0.001). Fat deposition, serum leptin, and insulin were significantly increased in offspring from control or high-fat fed dams if fed a high-fat diet from weaning to adulthood. However, only fat-fed offspring from fat-fed dams were hyperglycemic. Leptin receptor and neuropeptide Y (NPY) were also significantly increased in offspring exposed to excess saturated fat during gestation and into adulthood, whereas NPY1 receptor was downregulated. Signal transducer and activator of transcription 3 mRNA level was significantly higher in offspring from high-fat-fed dams compared with controls; however, no change was detected in cocaine and amphetamine-regulated transcript or suppressor of cytokine signaling 3. A significant reduction in
phosphatidylinositol 3-kinase regulatory subunit (p85α) mRNA (P < 0.001) and protein levels (P < 0.006) were coupled to an upregulation of protein kinase B mRNA (P < 0.014) in offspring from high-fat-fed dams transitioned to chow food. In contrast, p85α subunit mRNA was significantly increased in offspring from high-fat fed dams weaned to the high-fat diet (P < 0.05). These data support the hypothesis that early life exposure to excess fat is associated with changes in hypothalamic regulation of body weight and energy homeostasis and that postweaning diet influences the development of metabolic dysfunction and obesity.

Endoplasmic Reticulum (670 – 684)

670/B617
Rab32 Inhibits Apoptosis and Disrupts Calnexin Targeting to the Mitochondria-Associated Membrane.
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The mitochondria-associated membrane (MAM) has emerged as an endoplasmic reticulum (ER) signaling hub that accommodates ER chaperones. At the MAM, these chaperones control ER homeostasis, but also play a role in the onset of ER-stress mediated apoptosis. These opposing roles of MAM-localized chaperones suggest the existence of mechanisms that regulate the composition and the properties of ER membrane domains. Our results now localize the GTPase Rab32 to the ER and identify this protein as a novel ER domain orchestrator. Consistent with such a role, active Rab32 disrupts MAM retention of the chaperone calnexin. Furthermore, Rab32 determines the targeting of PKA to membranes of the ER and mitochondria. This function of Rab32 controls the phosphorylation of Bad on serine 155 and of Drp1 on Serine 656. Through a combination of its functions as a PKA-anchoring protein (AKAP) and a regulator of MAM composition, Rab32 over-expression inhibits apoptosis.

671/B618
Seipin, but Not a Point Mutant That Results in Lipodystrophy, Is a Component of a Discrete Proteinaceous Complex.
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Seipin is an ER integral membrane protein that is important for cytoplasmic lipid droplet assembly and/or maintenance; droplet morphology is severely altered in yeast harboring a seipin gene disruption, and patients with mutated seipin (notably A212P) have an absence of subcutaneous adipose tissue and develop fatty liver and insulin resistance. (Other seipin mutations cause the neuropathic Silver syndrome.) Human seipin can complement the yeast null strain. We had observed by fluorescence microscopy that seipin-mCherry forms puncta at the junction of ER and droplets, suggesting that several seipin molecules associate in a complex (Szymanski et al., PNAS, 2007). To learn more about the nature of the seipin complex, we subjected to velocity sedimentation detergent-solubilized yeast ER membranes containing seipin tagged in the genome with 13 copies of the myc epitope (seipin-myc13). In a detergent glycerol gradient, seipin-myc13 behaves as a ~220 kDa protein (monomer MW=53 kDa) with respect to soluble protein standards. Overexpressed protein behaves similarly, and overexpressed seipin-mCherry or overexpressed untagged protein also are found in large discrete complexes. We employed the method of Clarke and Smigel (Meth Enzymol 1989) to assess the extent of detergent binding in the complex and to determine the MW of the proteinaceous component. Using H₂O- and D₂O-sucrose gradients and S500 gel filtration, we determined that seipin-myc13 is in a 498±6 kDa protein complex. The Stokes' radius of the protein-detergent complex was 113 Å (similar values using cholate or Triton X-100), indicating an elongated shape, probably a disk, in the membrane. The extrapolated S²₀,ₐ value of the protein-detergent complex was 11.1. Interestingly, in cells expressing a point mutation in seipin-myc13, G225P, orthologous to the A212P human mutation, the complex was smaller: The protein component was 316±6 kDa (S²₀,ₐ=8.0 for the protein-
Whether seipin is in a homo-oligomeric or hetero-oligomeric complex is not yet clear. These results suggest that assembly of the correct seipin complex is important for the function of seipin in droplet biology, and in the generation of normal adipose tissue in humans.

672/B619

**Cadmium-Mediated Rescue from ER-Associated Degradation Induces Expression of Its Exporter.**

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Endoplasmic reticulum-associated degradation (ERAD) ensures the secretion of correctly folded and assembled proteins, which is necessary for the prevention of toxic accumulation of aberrant proteins. Our data showed that in addition to quality control, cells utilize ERAD for regulating the expression of a plasma membrane protein in response to cellular signals. Turnover of Pca1, a functional and naturally expressing cadmium efflux pump in yeast Saccharomyces cerevisiae, is mediated by ERAD via a degradation signal that can be masked by cadmium. A cysteine-rich amino-terminal degron rapidly targets the constitutively expressing Pca1 to the ERAD pathway. However, in the presence of cadmium, direct sensing of metal ions within the degradation signal induces its conformational change, which leads to an escape of Pca1 from ERAD. This substrate-mediated rescuing from destruction at the ER allows secretion of Pca1 for cadmium extrusion at the cell surface. Given that delicate control of homeostatic acquisition and detoxification of metal ions is a vital biological process, this mode of regulation represents a unique interaction between organism and toxic metal ion. Yeast has likely evolved this regulatory mechanism for a rapid cellular response to environmental changes. The ERAD of a significant portion of the secretory proteome may not be only attributed to a failure of correct folding. Rather, secretion versus degradation at the ER might be coupled through cellular cues as demonstrated for Pca1.

673/B620

**Hog1 MAP Kinase Coordinates a Late-Phase Endoplasmic Reticulum Stress Response Pathway in Budding Yeast.**

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In eukaryotic cells, nascent secretory proteins are translocated into the endoplasmic reticulum (ER), which promotes their proper folding and maturation before allowing them to be targeted to their final destinations. Under certain environmental or developmental conditions, unfolded proteins can overwhelm the protein folding capacity of the ER, thus leading to ER stress. Upon sensing ER stress, the Unfolded Protein Response (UPR) signaling pathway rapidly activates the transcription of genes that help expand the ER's protein-folding capacity. However, very little is known about how the cell responds if ER stress continues to persist after this initial wave of gene activation. We show that Hog1, a MAP kinase known to function in the osmotic stress response, coordinates a multi-faceted response to persistent ER stress. Under conditions of prolonged ER stress, Hog1 becomes phosphorylated through a unique mechanism, overlapping but distinct from the Hog1 activation mechanism of the osmotic stress response. This activation of Hog1 directly enhances the rate of recovery within the ER lumen. Upon phosphorylation, Hog1 is first translocated into the nucleus where it regulates a unique transcriptional program. Subsequently, although phosphorylation levels remain high, Hog1 returns to the cytoplasm. This step distinguishes the late-phase ER stress response from the osmotic stress response, in which Hog1 phosphorylation correlates strictly with nuclear localization. Upon returning to the cytoplasm, Hog1 activates autophagy by enhancing the production of Atg8, a critical autophagy protein. HOG1-mediated Atg8 production occurs at a post-transcriptional level through Hog1’s cytoplasmic target, Rck2. Mammalian cells also elicit a unique response to persistent ER stress, in this case marked by the specific activation of apoptotic pathways. Thus, understanding the distinction between early and persistent ER stress may be important for understanding the human pathologies that result from downstream effects of ER stress.

**674/B621**

**An Essential Role for XBP-1 in Host Protection against Immune Activation in C. elegans.**

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The detection and compensatory response to the accumulation of unfolded proteins in the endoplasmic reticulum (ER), termed the Unfolded Protein Response (UPR), represents a conserved cellular homeostatic mechanism with important roles in normal development and in the pathogenesis of disease. The IRE-1-XBP-1 pathway is a major branch of the UPR that has been conserved from yeast to human. XBP-1 is required for the differentiation of the highly secretory plasma cells of the mammalian adaptive immune system, but recent work also points to important reciprocal interactions between the UPR and other aspects of immunity and inflammation. We have been studying innate immunity in the nematode *Caenorhabditis elegans*, having established a key role for a conserved PMK-1 p38 mitogen-activated protein kinase (MAPK) pathway in mediating resistance to microbial pathogens. Here, we show that during *C. elegans* development, XBP-1 has an essential role in protecting the host during activation of innate immunity. Activation of the PMK-1 p38 MAPK-mediated immune response by infection with *Pseudomonas aeruginosa* induces the XBP-1-dependent UPR. We find that XBP-1 is required for development and that loss of XBP-1 activity leads to lethality. We find that this developmental phenotype is dependent on activation of the innate immune response. Our data establish innate immunity as a physiologically relevant and developmentally critical initiator of ER stress in *C. elegans* and suggest that an ancient, conserved role for XBP-1 may be to protect the host organism from the detrimental effects of mounting an immune response.

**675/B622**

**Domain Regulation of Endoplasmic Reticulum-Associated Degradation.**

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Gp78 is an E3 ubiquitin ligase that targets proteins for proteasomal degradation through the endoplasmic reticulum-associated degradation (ERAD) pathway. The endoplasmic reticulum (ER) is organized into functionally distinct membrane domains, however ER domain regulation of ERAD functions remains poorly understood. Here, we show that gp78-mediated ubiquitylation is initiated in a peripheral ER domain to which gp78 recruits the p97 ATPase. However, derlin-1, derlin-2 and GFP-Sec61β, all involved in retrotranslocation of ERAD substrates, are localized to a central, juxtanuclear ER domain. Ubiquitylated protein accumulation in the central ER upon proteasome inhibition identifies this ER domain as the site of retro-translocation. Transfer of ubiquitylated substrate from the peripheral to the central ER is dependent on ubiquitin chain elongation, interaction with the ubiquitin-binding CUE domain and p97 recruitment. Spatial segregation of gp78 ubiquitin ligase activity from proteasomal degradation defines novel ER domain regulation of ERAD function. Supported by CIHR Grant MT-15132.

**676/B623**

**Yip1A Regulates Mammalian Endoplasmic Reticulum Structure.**

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The mechanisms that regulate cisternal and tubular endoplasmic reticulum (ER) membrane network morphology in animal cells are not fully understood. Here we report that Yip1A, a highly conserved multi-pass integral membrane protein that cycles between the ER and Golgi, is required for the maintenance of normal ER network structure and organization in mammalian cells. Yip1A depletion caused collapse of the typically dispersed ER network into multiple micron-sized concentric whorls of stacked ER tubules and sheets. The whorled ER phenotype was a specific consequence of Yip1A silencing because it was rescued by expression of wild type but not mutant Yip1A. Of the established Yip1A binding partners, DP1, a protein restricted to the tubular ER and implicated previously in ER membrane curvature, is a reasonable candidate for mediating its membrane dispersal function. Several observations suggested that Yip1A
functionally opposes DP1 and DP1L1, as well as PRA2, a membrane protein also localized to the tubular ER and predicted to possess a membrane topology similar to DP1. First, high level DP1, DP1L1 or PRA2 over-expression resulted in ER whorls similar to those induced by Yip1A loss. Second, modest DP1, DP1L1 or PRA2 over-expression enhanced the Yip1A knock down phenotype. Third, Yip1A bound both DP1 and DP1L1. These data suggest that Yip1A maintains a dispersed ER reticulum through its interactions with a special class of tubular ER membrane localized membrane proteins. Finally, the structural reorganization of the ER reticulum in response to Yip1A loss caused a marked delay in coat protein II (COPII)-mediated protein export. Thus ER network dispersal by Yip1A may provide a means of controlling the flow of cargo out of the ER.

677/B624
The Mechanism of Ptc1p-Dependant Cortical ER Inheritance in Saccharomyces cerevisiae.
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In the yeast Saccharomyces cerevisiae, the peripheral Endoplasmic Reticulum (ER) forms a cortical network. It is inherited from mother to daughter cells in multiple stages including the extension of linear tubules along the mother-bud axis, anchorage at the bud tip, distribution along the bud periphery, and finally formation of a polygonal network in the daughter cell. Ptc1p, a type 2C serine/threonine protein phosphatase has been implicated at the late stages of cortical ER inheritance through regulation of the Cell Wall Integrity (CWI) MAP kinase, Slt2p. We have shown that inactivation of Slt2p by Ptc1p occurs concomitantly with the spread of cortical ER from the bud tip to the periphery after an ER tubule has reached the bud tip. Moreover, genetic analysis has shown that the pool of Slt2p controlling ER inheritance requires the CWI pathway scaffold, Spa2p, for its retention at the bud tip, while, the nuclear pool of Slt2p doesn’t contribute to its function in ER inheritance. We also show that the PI4P5-kinase, Mss4p, is an upstream activator of this pool of Slt2p. A family of cell surface sensors, Wsc1p or Mid2p, which is known to mediate Slt2p activation upon certain stresses, is not involved in Ptc1p regulation of ER inheritance. Rom2p acts downstream of the cell surface sensor in the CWI pathway, however, its localization at the tips of small buds is not affected in ptc1 deletion mutant. These observations together suggest a model in which Ptc1p acts well downstream of Wsc1p, Mid2p and Rom2p in the regulation of the CWI pathway by directly dephosphorylating Slt2p. Additionally, Ptc1 has also been indicated in vacuole and mitochondria inheritance. However, our data imply that different molecular mechanisms underly the inheritance of different organelles. While mitochondrial inheritance requires the down regulation of Slt2p, like ER inheritance, Spa2p is not needed for the activity of Slt2p and loss of Mss4p function does not suppress the mitochondrial inheritance defect of ptc1 mutant cells. In contrast, down regulation of Slt2p is not needed for the inheritance of vacuoles, but the loss of Mss4p function suppresses the vacuolar inheritance defect in ptc1 deletion cells.

678/B625
Role of Reticulons and Phospholipid Biosynthesis in Maintaining ER Tubules.
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The endoplasmic reticulum (ER) forms a highly dynamic network of tubules. In the yeast S. cerevisiae, the tubular portions of the ER are mostly near the cell periphery while the perinuclear ER is sheet-like. Recently we found that a family of membrane proteins required to maintain the tubular structure of the ER, the reticulons (Rtn1p, Rtn2p) and reticulon-like proteins (DP1/Yop1p), are localized exclusively in the peripheral ER. Our lab is interested in how these proteins shape the ER in S. cerevisiae. We found that these proteins must oligomerize to stabilize membrane tubules. To better understand the mechanism of their localization, we isolated mutations in Rtn1p that are distributed equally in the ER. Surprisingly, these mutants are still partly functional, because they are able to restore the sheet-like ER in a strain lacking tubule-shaping proteins. We demonstrated that these mutations decrease the ability of Rtn1p to oligomerize, suggesting that
the oligomerization of Rtn1p with itself or with other proteins is important to keep the protein in the highly curved membranes of the ER tubules. Interestingly, the mobility of Rtn1p within the ER membrane is affected by ATP-depletion, suggesting that Rtn1p oligomerization must be highly regulated. We would also like to understand why cells need tubular ER since cells lacking the reticulons and YOP1 have no apparent phenotype despite the absence of tubular ER. We identified mutations in genes that are synthetically lethal with deletion of RTN1, RTN2 and YOP1. Two of these have defects in the same lipid biosynthesis pathway. Loss of these proteins leads to overproduction of phospholipids, which affects ER structure. Interestingly, overexpression of Rtn1p in these strains restores tubules. These findings suggest that the phospholipids level in the ER and reticulons are required for proper ER function.

679/B626

Usa1 Is a Key Regulator of the HRD1 ERAD Ligase.

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ER-associated degradation (ERAD) is a highly conserved process by which misfolded proteins are eliminated from the ER in order to prevent deleterious stress caused by their accumulation. Ubiquitin ligases in the ER ubiquitinate misfolded proteins, which are ultimately degraded in the cytosol by the 26S proteasome. Hrd1 is a conserved ERAD ubiquitin ligase with a multispansing transmembrane anchor and a catalytic RING motif in the cytoplasm. Hrd1 is part of the HRD complex required for the identification, ubiquitination, retro-translocation, and proteasomal degradation of a wide variety of luminal and integral membrane ERAD substrates. Usa1 is the newest and least characterized member of the HRD complex, inviting our study of its roles in ERAD and HRD function. Usa1 is required for optimal function of the HRD complex, promoting degradation of both luminal and membrane-associated substrates. Although the action of the HRD pathway is under intense study, little is known about the mechanisms by which this broadly active ligase is regulated to ensure efficient activity without cellular detriment. We now show that Usa1 is a key regulator of both Hrd1 levels and activity. When Hrd1 levels exceed those of Hrd3, Hrd1 undergoes rapid self-ubiquitination and degradation, limiting its levels to ensure HRD complex stoichiometry. Usa1 and specifically its Ubl domain are essential for Hrd1 self-destruction, by promoting trans-ubiquitination of one Hrd1 molecule by another Hrd1, in a variant of ERAD entirely specific for the Hrd1 protein. Because unregulated Hrd1 has been observed to be toxic to cells, we investigated Usa1’s role in this aspect of Hrd1 regulation. In addition to controlling Hrd1 stability (and hence, levels) Usa1 has an independent action in limiting Hrd1 activity to ERAD substrates, thereby protecting the cell from overactive ERAD in two ways. Thus Usa1 exhibits a dual role with respect to Hrd1 function - it governs the levels of Hrd1 and is required for auto-ubiquitination of Hrd1 in the absence of Hrd3, and has an additional role in modulating the activity and specificity of Hrd1. Thus, Usa1 is a key player in HRD ligase dynamics and function.

680/B627

A Conserved Sterol-Dependent Interaction with Insig Mediates Destruction, Or Not.

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Insig proteins are central regulators of sterol balance. They modulate the uptake and synthesis of sterols through interactions with Scap (bound to the transcription factor SREBP) and HmgCoA Reductase (HMGR). HMGR catalyzes the production of mevalonate, the rate-limiting step in isoprene and subsequent sterol synthesis. Lanosterol-mediated binding of Insig to HMGR drives the HMGR degradation, allowing feedback-regulated degradation of HMGR. The sterol-dependent Insig-HMGR interaction requires the sterol-sensing domain of HMGR. In yeast, there are two Insigs, Nsg1 and Nsg2 (pronounced “insig”). Both interact with Hmg2. Like mammalian HMGR, yeast Hmg2 undergoes feedback-regulated degradation in response to FPP levels, a sterol pathway intermediate. In contrast to mammals, the Hmg2 degradation is blocked by Nsg1
binding. This opposite effect may represent divergent requirements for the yeast Insig-HMGR interaction. Alternatively, a conserved interaction is affecting a distinct output as is fitting the specific biology of the organism. To test this we asked if a sterol molecule is required. We used drugs to block individual steps in sterol biosynthesis and then assayed the interaction. It is sterol-dependent and the responsible molecule is lanosterol, the same sterol used in mammals. Blocking lanosterol synthesis compromises the Nsg1/Hmg2 interaction but blocking a step downstream preserves the interaction. Thus both mammalian and yeast Insig-HMGR interactions integrate sterol levels with HMGR levels. Why is yeast Hmg2 stabilized by Nsg1? The hook-up between this integration point (Insig-HMGR interaction) and downstream events is tailored to biological context. Molecular oxygen is universally required for sterol synthesis. While mammalian cells are bathed in oxygenated blood, yeast may experience low/no oxygen. Since oxygen limits sterol synthesis and sterol levels are sensed by the Nsg1/Hmg2 interaction, perhaps the interaction is regulated by oxygen. We have tested this idea. Hmg2 and Nsg1 levels are low in anoxia relative to normoxia. In this way Hmg2 levels are calibrated for the production of essential isoprenes (early sterol pathway molecules), but not downstream sterols, when oxygen is limiting in yeast.

681/B628
Live Cell Imaging of CD3delta Retrotranslocation.
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Many misfolded and unassembled secretory proteins are retrogradely moved from the ER into the cytosol for proteasomal degradation by a process known as ER-associated degradation (ERAD). Here, photoactivatable GFP (PAGFP) and a dual-fluorescence labeling approach have been employed to dissect the retrotranslocation pathway of the transmembrane T cell receptor subunit, CD3delta. CD3delta-PAGFP molecules “switched on” by photoactivation exhibited a half-life of ~2h irrespective of intracellular protein expression levels. The visible molecules were highly mobile throughout the ER with no significant cytoplasmic pool, indicating that membrane sorting in the ER, rather than retrograde translocation and proteasomal degradation, is the rate-limiting step for ERAD trafficking of CD3delta. Use of dual-labeled YFPCD3deltaCFP further revealed the fate of the CD3delta pool dislocated from the ER membrane. Upon inhibition of the proteasome, YFPCD3deltaCFP diffused within the cytosol and the nucleoplasm at dynamics significantly slower than YFP alone. However, no detectable aggregates were formed by the dislocated pool, which was rapidly degraded upon reestablishment of proteasomal activity. Together, these results show that retrotranslocated CD3delta, if not degraded immediately, remains soluble and capable of entering the nucleus.

682/B629
Interactions among Endoplasmic Reticulum-Associated Degradation (ERAD) Complex Components Dynamically Change in Response to ER Stress In Vivo.
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Accumulation of misfolded proteins in the endoplasmic reticulum (ER) causes a cellular condition called ER stress. Herp is one of the representative proteins whose expression is induced under the condition. Herp contains 391 amino acid residues including an N-terminal ubiquitin-like domain and two contiguous transmembrane domains, and is embedded in the ER membrane. Although the molecular function of Herp remains unknown, recent studies suggested its contribution to the ER-associated protein degradation (ERAD) system. ERAD is a mechanism to deal with the undesirable proteins in the ER, where a number of proteins are involved. In the present study, we have generated Herp-deficient mice and analyzed the endogenous ERAD complexes. In the wild-type mouse liver, the levels of Herp, Derlin-1, Derlin-2, Derlin-3, HRD1, gp78, and VIMP were predictably elevated after the intraperitoneal injection of tunicamycin. In the Herp-deficient mice, the levels of these proteins were higher than those in the wild-type mice, suggesting that the deficiency of Herp caused sensitiveness to the experimental ER stress
condition. We have identified at least three distinct ERAD complexes. In the normal condition, Herp formed a complex with Derlin-2 and HRD1 (Complex Ia). In the ER stress condition, Derlin-3 was dramatically expressed and added to the complex, where the interactions among these proteins drastically altered (Complex Ib). In addition, a protein complex containing Derlin-1, Derlin-2, VIMP, and p97 appeared only in the ER stress condition (Complex II). In the Herp-deficient mice, the ER stress-dependent interactions among Derlin-1, Derlin-2, VIMP, and p97 were observed just like in the wild-type mice, suggesting that Herp does not affect the formation of Complex II. In contrast, the interaction between Derlin-2 and HRD1 in Complex Ia was significantly reduced as compared with in the wild-type mice, suggesting that Herp should be important for the formation of the complex. Taken together, our findings indicated that the ERAD complexes are dynamically changed in response to ER stress, where Herp may have a significant role.

683/B630
Catecholamine-Induced Apparent Fragmentation of Lipid Droplets in Adipocytes.
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The most characteristic organelle of adipocytes is the lipid droplet (LD), in which triglyceride (TG) is stored. Numerous studies have revealed that LDs act as highly dynamic organelles that play a crucial role in regulating energy homeostasis rather than as an inert reservoir for storing TG; in other words, mobilization of TG stored in LDs is also a major function of adipocytes. To date, the complicated molecular processes involved in lipolysis have been extensively studied; in contrast, studies on morphological changes of LDs during lipolysis are less. In the present study, we performed time-lapse microscopy of adipocytes stimulated with 1 × 10^-7 M norepinephrine to investigate morphological changes of LDs during lipolysis. The study used in vitro-differentiated adipocytes from stromal-vascular cells harvested from the mesenteric adipose tissue of rat. The results clearly show that the existing LDs shrank considerably and a part of them subsequently disappeared from immediately to 8 h after norepinephrine stimulation. However, such shrinking of the LDs did not occur in all adipocytes. Adipocytes in which the LDs were preserved or enlarged were also observed. Interestingly, although catecholamine stimulation would promote the release of FFA and glycerol from adipocytes, numerous small LDs (~1 μm) were newly developed in almost all the cells, including undifferentiated ones. Similar shrinking and development of LDs were also observed in adipocytes stimulated with 1 × 10^-7 M isoproterenol. Immunofluorescence imaging reveals that activation and localization of hormone-sensitive lipase (HSL), but not adipose triglyceride lipase, on the surface of LDs are necessary for their shrinking. In summary, the simultaneous shrinking and development of LDs yield apparent fragmentation and dispersion of LDs in adipocytes stimulated with catecholamine.

684/B631
Trafficking of Endogenous Viral Antigens from Endoplasmic Reticulum-Derived Compartments to Phagosomes Contributes to MHC Class I Presentation.
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Membrane trafficking events participate in several key aspects of cellular function, including immune defense mechanisms. Indeed, the formation of degradative organelles such as phagosomes and autophagosomes requires membrane rearrangements that imply complex membrane trafficking events. The endoplasmic reticulum (ER) compartment has been proposed to be a source of membrane for the biogenesis of both phagosomes and autophagosomes. In this study, we monitored the potential interaction between ER and phagosomes by following the trafficking of the glycoprotein B (gB) of Herpes simplex 1 (HSV-1) present in the ER of infected cells. Formation of latex bead-containing phagosomes in infected macrophages allowed us to observe ER-phagosome interactions during HSV-1 infection and the concomitant transfer of gB to phagosomes. The transfer of gB to phagosomes improves the processing and presentation of this
antigen on MHC class I molecules. Furthermore, our results indicate that phagosomes interact with autophagosomes highly enriched with gB, a process that also stimulates the MHC class I presentation of this antigen. Thus, membrane trafficking events involving ER-derived compartments and phagosomes enhance the ability of macrophages to promote CD8+ T cells activation.

Chloroplasts, Mitochondrial and Peroxisomes (685 – 706)

685/B632
Spatial and Temporal Regulation of Myosin-Driven Peroxisome Partitioning in Yeast.
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Cells are equipped with a variety of cytoskeletal motors that drive intracellular motility. In Saccharomyces cerevisiae, the class V myosin motor Myo2p propels the movement of most organelles. Organelle-specific receptors for Myo2p present on the surface of various cargoes dictate the timing of Myo2p attachment to organelles to control their motility. How these receptors are regulated to ensure coordinated delivery of organelles is for the most part unknown. We recently identified Inp2p as the peroxisome-specific receptor for Myo2p. Here we delineate the region of Myo2p devoted to binding peroxisomes and show that this region partially overlaps the region that binds secretory vesicles. Using mutants of Myo2p specifically impaired in binding peroxisomes, we dissect cell cycle-dependent and peroxisome partitioning-dependent mechanisms of Inp2p regulation. We find that total Inp2p levels are controlled by peroxisome inheritance, as Inp2p aberrantly accumulates and decorates all peroxisomes in mother cells when peroxisome partitioning is abolished. We also find that Inp2p is a phosphoprotein whose level of phosphorylation is coupled to the cell cycle irrespective of peroxisome positioning in the cell. Our findings point to the existence of several regulatory mechanisms that control the activity of organelle-specific receptors for molecular motors in response to both organelle positioning and cell cycle progression to ultimately achieve an equidistribution of compartments between mother and daughter cells.

686/B633
Phosphorylation-Dependent Trafficking of Peroxisome Proliferation Protein PEX11 from Endoplasmic Reticulum to Peroxisomes Controls Peroxisome Abundance.
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Peroxisomes are highly dynamic organelles that divide continuously in growing cell cultures and expand extensively in lipid-rich media. Peroxisome population control is achieved in part by Pex11p-dependent regulation of peroxisome size and number. Although the production and levels of Pex11p in yeast are tightly linked to peroxisome biogenesis by transcriptional regulation of the PEX11 gene, it remains unclear if and how Pex11p activity could be more rapidly modulated. Here we report the reversible phosphorylation of Saccharomyces cerevisiae Pex11p in response to nutritional cues and delineate a mechanism for phosphorylation-dependent activation of Pex11p through the analysis of phosphomimicking mutants. In fatty acid-grown cells, Pex11p is synthesized but spatially segregated from peroxisomes in a predominantly endoplasmic reticulum (ER)-bound state. A glucose stimulus causes the phosphorylation of Pex11p and its trafficking from the ER to peroxisomes. Pex11Ap and Pex11Dp mimicking constitutively dephosphorylated and phosphorylated forms of Pex11p, respectively, show differential association with the ER and are impaired in trafficking, which correlate with decreased levels of Pex11Ap, increased levels of Pex11Dp, and changes in peroxisome abundance. By analyzing wild-type Pex11p and Pex11Dp in a dose-independent manner, we demonstrate that
phosphorylation of Pex11p causes its activation and peroxisomal hyperproliferation. This work provides the first evidence for regulation of organelle dynamics by phosphorylation-controlled protein trafficking.

687/B634  
**Dictyostelium discoideum** Dynamin B Participates in Peroxisomal Division and Cell Adhesion.  
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 Dictyostelium discoideum (Dd) has five dynamins, dynamin A, dynamin B and the dynamin-like proteins Dlp A, Dlp B and Dlp C. Depletion of each of the dynamin-like proteins in Dd results in abnormal cytokinesis. Disruption of Dd dynamin A affects cytokinesis, endocytosis and organelle morphology. Here we describe the characterization of Dd dynamin B. Dynamin B contains an N-terminal sequence that targets it to mitochondria where processing of the protein takes place. We used a molecular genetic approach to manipulate the production levels of dynamin B in combination with a fluorescent tagging approach to visualize dynamin B localization, organelle morphology, and cytoskeletal filament dynamics. Dynamin B null cells and cells overproducing the protein do not show any obvious morphological alterations. They display normal uptake of fluid-phase markers, grow normally in the presence of axenic media, and form normal fruiting bodies during development. However dynamin B-depleted cells show a marked increase in the size of peroxisomes concomitant with a decrease in their number. A reduced tolerance of dynamin B-depleted cell towards the presence of long chain fatty acid indicates that peroxisome function is compromised. Additionally, we observed that phagocytosis is affected by changes in the expression level of the dynamin B gene. The absence of dynamin B correlates with increased cell to substrate and cell to cell interactions. Our results link the function of dynamin B to roles in the biogenesis and maintenance of peroxisomes and cell adhesion in *D. discoideum*.

688/B635  
**Ectopic PEX11β Alters Peroxisomal Gene Expression during Xenopus laevis Development.**  
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Peroxisomes are ubiquitous organelles responsible for fatty acid metabolism and H2O2 degradation. The biogenesis of fully functional peroxisomes is achieved by the coordinated activity of cytosolic and membrane-tethered proteins termed peroxins (PEX proteins). The objectives of the present study are to characterize the expression of specific peroxisomal genes, and to examine the onset and distribution of functional peroxisomes during Xenopus laevis development. The temporal and spatial expression of peroxisome related genes was determined by RT-PCR and whole-mount in situ hybridization, respectively, while the localization of functional peroxisomes was evaluated by microinjection of GFP tagged with a peroxisome targeting signal in early embryos. Five peroxisomal genes (PMP70, Catalase, PEX3, PEX5 and PEX11β) all showed increasing levels of expression as development proceeded. These genes had similar localization patterns in embryos at developmental stages 10, 20, 30 and 40, showing predominant anterior and dorsal expression. Microinjection of peroxisome targeted GFP demonstrated that at stage 10 no punctate GFP was visualized, suggesting no functional peroxisomes. However, functional peroxisomes were found at stage 20, and their number increased in later developmental stages in anterior and dorsal structures. Furthermore, microinjection of PEX11β (a known inducer of peroxisome division in yeast) into early embryos, followed by immunohistochemical analysis, was used to reveal whether PEX11β has a homologous function of increasing peroxisome numbers during X. laevis development. Microinjection of PEX11β mRNA resulted in the formation of peroxisomes by stage 10 as judged by use of a PMP70 antibody. Taken together, these results indicate that during X. laevis development
development, the formation of functional peroxisomes begins at stage 20 in anterior and dorsal structures. PEX11β may play a key role in peroxisome biogenesis, as overexpression of PEX11β induces the earlier formation of peroxisomes during X. laevis development.

689/B636
Molecular Interrogation of the Role of the Endoplasmic Reticulum in Peroxisome Biogenesis.
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The role proteins interactions and the resulting complexes play in regulating organelle biology are of fundamental importance in understanding cell biology at both reductionist and systems level. The hierarchical flow of information that enables organelle architecture, abundance and functions, although presumably cyclical with respect to the life cycle of a given cell type, are prone to genetic and environmental perturbations. These perturbations, as carried out in laboratory settings, enable systematic delineation of the underlying mechanisms that cell utilize in establishing homeostasis and evolutionary advantage. The peroxisome system in yeast Saccharomyces cerevisiae cells can be viewed as a good example of such a system. We employed a top-to-bottom approach to identify, protein complexes for Pex29 and Pex30, previously characterized as integral membrane proteins localized to peroxisomal membrane (peroxins). Unlike earlier efforts that enriched peroxisome fractions prior to immuno-isolation of peroxin complexes, we optimized the protocol to perform immuno-isolation from whole cell extracts so as to globally probe for all potential interaction partners for peroxins. This strategy is especially important given that de novo biogenesis of peroxisomes initiates on the ER template. We used biochemical isolation of protein complexes followed by mass-spectrometry based proteomics to identify the individual proteins in combination with yeast molecular genetics and dynamic quantitative microscopy. These studies have revealed a role for ER resident proteins in the emergence of peroxisomes from the secretory system. Taken together, these studies will further our knowledge regarding the role these protein complexes play in peroxisome biogenesis.

690/B637
A Strong Association of 3β-Hydroxysteroid Dehydrogenase Type II (3βHSD II) with the Mitochondrial Membrane Is Essential for Steroidogenic Activity.
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Protein trafficking into a specific mitochondrial compartment is dependent on the proteins present at the outer and inner membranes. Mitochondrial human type 2 3β-hydroxysteroid dehydrogenase (3βHSD II) is a key steroidogenic enzyme, in the adrenals, gonads and brain that catalyzes, in three independent steps the production of steroid hormones: progesterone, aldosterone, cortisol, testosterone and estradiol. Thus any abnormal function will result in a fatal error in steroid metabolism. To explore the role of mitochondrial association with enzyme action, 3βHSD II was synthesized in a cell-free transcription system labeled with 35S-methionine and incubated with isolated mitochondria from steroidogenic tissues. The import reaction shows that 3βHSD II was associated with the mitochondrial membrane, since extraction with Na2CO3 retained the imported fraction in the pellet. To determine the nature of association with the mitochondrial membrane, we extracted with nonionic, anionic and zwitterionic detergents: digitonin, lauryl maltoside, taurodeoxycholic acid and CHAPS. These detergents yielded a high molecular weight complex, of approximately 500 KDa when analysed through a 3-16% gradient native PAGE. The integration of lauryl maltoside associated complex was stronger than the zwitterionic or anionic detergents: digitonin, lauryl maltoside, taurodeoxycholic acid and CHAPS. These detergents yielded a high molecular weight complex, of approximately 500 KDa when analysed through a 3-16% gradient native PAGE. The integration of lauryl maltoside associated complex was stronger than the digitonin isolated complex when proteolysed with proteinase K. The ionic detergent deoxycholic acid didn’t form any complex but taurodeoxycholic acid was very effective at isolating a 3βHSD II complex, because of its long aliphatic sidechain that contains a sulfonyl group. The zwitterionic, CHAPS, also formed a strong complex with 3βHSD II associated proteins because of a putative membrane-association loop of 26 amino acids present in the 3βHSD II, as determined by mutagenesis and computer modeling. The biological activity with lauryl maltoside extracted complex was higher than with the zwitterionic or anionic detergents. These experiments clearly
demonstrate a role of other mitochondrial proteins for the translocation of 3βHSD II into the mitochondrial membrane.

691/B638
Mechanism of Import of Cytochrome P450 Side-Chain Cleavage Enzyme (P450scc) into Mitochondria.
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Cytochrome P450 side-chain cleavage enzyme (P450scc), a key component of the mitochondrial P450 complex, cleaves the side chain of cholesterol to form the first steroid in the tissue pregnenolone. To catalyze this reaction, P450scc accepts a pair of electron from ferrodoxin and ferrodoxin reductase at the matrix side of mitochondria. So, any change in P450scc function results in gross error in steroid metabolism and is fatal for survival of all species. P450scc is a 521 amino acid protein with a possible cleavage at 39th amino acid, as determined by indirect methods long time ago. The mechanism of action, import and processing of P450scc into the mitochondrial matrix remained unknown. Mitochondrial import of cell-free synthesized P450scc, labeled with 35S methionine cleaved it to an intramitochondrial smaller form. The shorter fragment remained in the pellet, suggesting a membrane association; but it was not integrated into the lipid membrane as it remained in the solution when extracted with sodium carbonate. We next determined the role of N- and C-terminal amino acids sequences for its mitochondrial import and translocation. We built several constructs, synthesized in cell-free system labeled with 35S methionine and imported into isolated mitochondria. The deletion of upto 50 AA from the C-terminus had no effect on import. Similarly, addition of extra 50 AA at the C-terminus showed unchanged import of P450scc, suggesting the C-terminal AA has no role on its translocation into mitochondrial compartment. Site directed mutagenesis of the amino terminal sequences from amino acids 35 to 50, did not affect the cleavage of P450scc. on the other hand internal deletion of 45 amino acids (Δ 15-60) showed no import, suggesting that the cleavage site is between 15-60 amino acids. A careful analysis of the mitochondrial import shows that the imported P450scc has two bands, suggesting that the cleavage sites are possible before 35th and after 50th amino acids. Thus the experiment clearly demonstrates a stepwise import and processing of P450scc into mitochondria.

692/B639
Promoter Independent Regulation of Aldosterone Synthase.
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Aldosterone controls blood volume by retention of sodium and release of potassium and hydrogen ions in the kidney. Aldosterone excretion can contribute to hypertension, which results from higher than normal blood pressure and is directly linked to coronary heart disease. Steroidogenic protein aldosterone synthase (AS) converts deoxycorticosterone to aldosterone in mitochondria. In order for AS to perform this function, the precursor form of the enzyme must be targeted to and translocated across the outer mitochondrial membrane (OMM) before arriving at its final destination. However, the mechanism of action has remained unknown. To understand the mechanism of AS translocation, we incubated cell-free synthesized radiolabeled AS with isolated adrenal mitochondria. We observed cleavage of precursor AS (57 kD) to a mature form (54 kD) that was protected from exogenous proteinase K activity, while uncleaved precursor was digested. This demonstrates that mature AS is translocated across the OMM, with cleavage occurring afterwards, and that precursor remains outside of the mitochondria. Translocation kinetics studies revealed that formation of the mature form occurs very rapidly, reaching a peak level within minutes. Once this peak is reached, processing is abruptly halted. In contrast, P450SCC (SCC), another steroidogenic protein localizing to the mitochondria, never reaches a maximum level of the mature form, alternatively showing a steady increase over time. In order to study AS activity, we transfected COS-1 cells with a fusion construct of AS and dihydrofolate reductase (DHFR). The construct was equally as active as wild type AS, and this activity was significantly reduced in the presence of methotrexate, which binds DHFR and prevents complete
translocation of the protein. This result confirms that translocation into the mitochondria is required for AS activity. In summary, we conclude that this promoter-independent regulation of translocation can potentially control the synthesis of aldosterone in response to cell stimulated expression of AS, thus preventing overproduction of aldosterone.

693/B640
Disrupting the Drosophila Delta-1-Pyrroline-5-Carboxylate Dehydrogenase Gene Causes Mitochondrial Defects.
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Delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH) is a mitochondrial matrix enzyme that catalyzes mainly the second step of proline degradation. Mutation of P5CDH in humans (called ALDH4A1) causes type II hyperprolinemia, a complex syndrome that includes mental disabilities and seizures in some patients (Geraghty et al., 1998, Hum. Mol. Genet. 7: 1411-1415). Two genes in Drosophila melanogaster display sequence similarities to the human ALDH4A1 gene. The protein encoded by Drosophila conceptual gene CG7145 has 62% sequence identity and 77% similarity of the human ALDH4A1 protein, while the protein encoded by the second gene, CG33092, shares 48% sequence identity and 66% similarity to the ALDH4A1 protein. To explore for possible symptoms of hyperprolinemia in Drosophila, we studied a mutant fly line CG7145 with a PBac transposon inserted into the CG7145 gene. The insertion introduces a stop codon in the coding sequence of CG7145 such that the protein product is 100 amino acids shorter than the full length protein. Heterozygous (CG7145/TM3) individuals develop normally, while homozygous (CG7145/CG7145) individuals display lethality during the second and third larval instar stages, and in the pupal stage. Transmission electron microscopy of CG7145 larval tissues showed swollen mitochondria with diameters approximately 1.5 times greater than those of mitochondria in control larvae. Mitochondrial matrices appeared devoid, while cristae appeared reduced in number. We believe this is the first demonstration that a mutation in a type P5CDH mitochondrial enzyme causes morphological defects in the mitochondria, perhaps explaining the complex phenotypes associated with type II hyperprolinemia.

694/B641
Histone H3 Identified in Mitochondria: Key to Evolution and Nuclear-Mitochondrial Coordination?
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The core histone proteins H2A, H2B, H3, and H4 are known to form nucleosomes with nuclear DNA, but are historically considered to be absent from mitochondria. The objective of this study was to determine if H3 is found in mitochondria. We found, by several different methods, that H3 is a dual-targeted protein, found in mitochondria as well as nuclei. Analyses of various H3 amino acid sequences with WoLF PSORT and MitoProt revealed mitochondrial targeting signals for all variants and all species of H3 sampled, and Western blots of Brassica oleracea (cauliflower) mitochondrial extracts were positive for H3. Tandem Mass Spectrometry analyses confirmed the Western blot data. Further Western blot analysis revealed N-terminal modification of mitochondrial H3 as compared to nuclear H3. We conclude that H3 is targeted to the mitochondria and that mitochondrial H3 has an N-terminal modification; and we hypothesize that since modifications of the core histone H3 (H3) are known to help control nuclear genes, future studies of the functions of mitochondrial H3 could lead to a greater understanding of the ability of a cell to synchronize nuclear and mitochondrial activities.

695/B642
Dynamics of Axonal Mitochondria Is Regulated by Myelination in the Central Nervous System.
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Neurons are highly polarized cells with a single long axon, many of which are ensheathed by myelin. Although mitochondria are essential for axonal function and are transported and distributed throughout the axons, the transport, turnover and general behavior of mitochondria in myelinated axons are poorly understood. In this study, we established an In Vitro model for live imaging of mitochondria in myelinated central nervous system (CNS) axons, and compared mitochondrial distribution and motility at internodal and nodal regions. Organotypic slice cultures of cerebellum were prepared from P10 Sprague-Dawley rats, and mitochondria-targeted DsRed were expressed in Purkinje neurons. After myelination, mitochondrial distribution and movement were observed by time-lapse imaging using confocal microscopy. After imaging, slices were fixed, stained for nodal and paranodal proteins, and the time-lapse images were superimposed on the nodal, paranodal and internodal regions of the same fibers. Many Purkinje cell axons were myelinated during the 14 days in culture, and some contained DsRed-labeled motile and stationary mitochondria. We found that motile mitochondria stopped or slowed down around the nodes of Ranvier, and the mean velocity was significantly lower around nodal areas when compared to internodal areas of the same axons. Stationary mitochondria localized in juxtaparanodal and nodal regions of myelinated axons, but were less frequently found in paranodal regions. When the slices were treated with TTX to examine the effect of voltage-dependent Na current at the nodes, the number of motile mitochondria increased, and mitochondrial velocity was similar at nodal and internodal segments of the same axon. Furthermore, the sizes of stationary mitochondria decreased around the nodes. These results demonstrate myelination regulates mitochondrial motility in CNS axons by rendering topographical variance of axonal molecules and electrical activity. The focal modulation of mitochondrial dynamics at the nodes of Ranvier would be essential for efficient maintenance of axonal homeostasis upon neuronal activation.

696/B643
Mitochondrial Fragmentation Is Required for Proper Mitotic Entry.
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DNA duplication and partition into daughter cells are tightly regulated by a series of cell cycle checkpoints during cell division. However, mechanisms on partitioning of cellular organelles remain largely unknown. In interphase, a highly interconnected tubular network of mitochondria is observed but it no longer exists in mitotic cells, showing rather fragmented mitochondria. Here, we addressed whether mitochondrial fragmentation is necessary for proper cell cycle progression and cellular function. Since mitochondrial fission and fusion determine the mitochondrial morphology, we suppressed the mitochondrial fission activity by depleting one of the fission modulator, hFis1 by using a shRNA system. Notably, cells lacking hFis1 showed significantly elongated mitochondria and in these cells, cell cycle progression into mitotic phase was significantly impaired. Few cells entered mitosis under time-lapse microscopy and mitotic accumulation of hFis1 RNAi cells was very limited by aceto-orcein staining. In these cells, cyclin B1 failed to accumulate and the phosphorylated form of histone H3 was not detected, indicating that mitochondrial fragmentation is indispensable for proper mitotic entry. When mitochondrial fragmentation is induced by double knockdown of hFis1 and Opa1, a component of mitochondrial fusion, the cells regained ability to enter mitosis. Moreover, besides of low cyclin B1, expressions of cyclin A, cyclin-dependent kinase 1(Cdk1), polo-like kinase 1 (Plk-1) and aurora B kinase are significantly low in hFis1-depleted cells. Interestingly, introduction of active Plk1 or FoxM1, but not of active cyclin B1/Cdk1, into hFis1 RNAi cells significantly restored the mitotic entry. Together, one of the key functions of mitochondrial fission would be mitochondrial reorganization at G2 phase that is essential for proper mitotic entry.

697/B644
An Ultrasensitive Dual Emission Mitochondrial Membrane Potential Probe for Monitoring Mito-Toxicity in Live Cells.
Mitochondrial membrane potential (MMP) is a key indicator of cell health, mitochondrial permeability transition and apoptosis. Loss of MMP is often associated with early stages of apoptosis. Collapse of the MMP coincides with the opening of mitochondrial permeability transition pores, leading to the release of cytochrome c into the cytosol. This triggers downstream apoptotic events. Therefore, depolarization is a good indicator of mitochondrial dysfunction, and is increasingly utilized in drug toxicity measurements. JC-1, a lipophilic cationic carbocyanine dye, is widely employed to detect MMP. However, poor water solubility of JC-1 makes it difficult to use, especially in image-based and fluorescence plate reader-based applications wherein complete removal of un-dissolved fluorescent particles from the assay solution is critical for obtaining high-quality data. Additionally, spectral compensation is somewhat problematic in flow cytometry-based applications. We describe a new water-soluble dual emission MMP probe with similar fluorescent properties and subcellular localization as JC-1 dye. The performance of this dye was compared to JC-1 using various effectors of MMP and several different cell lines. The assay was also validated with a broad range of fluorescence detection platforms, including wide-field fluorescence microscopy as well as flow cytometry and microplate-based cytometry. Overall, the new dye is at least 10-fold more sensitive to MMP loss, is more photostable and more soluble than JC-1. Potential applications of the new probe include preclinical drug safety assessment (ADME-Tox) using In Vitro cell culture models to aid in the drug development process, especially to differentiate among compounds and rank order their potency.

698/B645
MICS1 Is Involved in Maintenance of Mitochondrial Morphology and Apoptotic Release of Cytochrome C.
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Mitochondrial morphology dynamically changes in a balance of membrane fusion and fission in response to the environment, cell cycle, and apoptotic stimuli. Here, we report that a new mitochondrial protein, MICS1, is involved in mitochondrial morphology in specific cristae structures and the apoptotic release of cytochrome c from the mitochondria. MICS1 is an inner membrane protein with a cleavable presequence and multiple transmembrane segments, and belongs to the Bi-1 super family. MICS1 downregulation causes mitochondrial fragmentation and cristae disorganization, and stimulates the release of pro-apoptotic proteins. Expression of the anti-apoptotic protein Bcl-XL does not prevent morphologic changes of mitochondria caused by MICS1 downregulation, indicating that MICS1 plays a role in maintaining mitochondrial morphology separately from the function in apoptotic pathways. MICS1 overproduction induces mitochondrial aggregation, and partially inhibits cytochrome c release during apoptosis, regardless of the occurrence of Bax targeting. Thus, MICS1 may facilitate the tight association of cytochrome c with the inner membrane during apoptosis. Furthermore, under low serum condition in which MICS1 is upregulated, the apoptotic release of cytochrome c is delayed without significant changes in mitochondrial morphology, suggesting that MICS1 individually functions in mitochondrial morphology and cytochrome c release.

699/B646
Mitochondrial Characteristics Associated with Pluripotent Non-Human Primate Embryonic Stem Cells.
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While embryonic stem cells have fostered optimism that these pluripotent cells could represent powerful models of human development and disease, there has been little characterization of the
cellular properties of ES cells compared to differentiated cells. Mitochondrial properties were compared in three Rhesus monkey embryonic stem (ES) cell lines, ORMES 6, 7 and 22 to determine their suitability as biomarkers of stem cell function. All lines expressed the pluripotency markers Oct 4 and Nanog, and 100% of the cells examined had a considerable mass of active mitochondria, as viewed by Mitotracker red staining. Cells exhibited a ‘capped’ mitochondrial localization pattern in which mitochondria cluster tightly to one side of the nucleus in 92-98% of the cells examined, depending on cell line. Undifferentiated ES cells had a 2-4 fold lower ATP content/cell (range 0.0354 to 0.0671 pmoles ATP/cell) compared to a differentiated monkey fibroblast control (0.163 pmoles ATP/cell). Upon removal of growth factor (bFGF) and feeder support, which releases cells from the pluripotent state, ORMES 22 cells lost expression of Nanog, associated with dispersal of mitochondria throughout the cytoplasm and a significant increase in ATP content/cell (0.110 p moles ATP/cell, P = 0.006). These results suggest that mitochondrial localization and ATP content per cell may be valid biomarkers of pluripotency, and that changes in these biomarkers may signal differentiation.

700/B647

Docking Receptor Syntaphilin Is Required for Calcium-Dependent and KIF5-Miro-Mediated Immobilization of Axonal Mitochondria.

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The coordination of mitochondrial mobility with axonal physiology is crucial for proper neuronal development and synaptic function. Motile mitochondria are recruited to the stationary pool near synapses in response to elevated cytosolic Ca2+ and synaptic activity. The mechanisms as to how motile mitochondria are recruited to stationary pool in response to neuronal activity remain unknown. Recent advances in identifying syntaphilin as a mitochondrial docking receptor (Kang et al., Cell 2008) and KIF5 motor adaptor Miro as a calcium sensor arresting mitochondrial movement provide molecular targets for such regulation. Using snph KO mouse combined with time-lapse imaging we sought to address whether SNPH is required in Ca-dependent arresting of axonal mitochondrial movement. First, elevated cytosolic Ca2+ in axons by treating snph (+/+) neuron with 50 mM KCl and 10 μM FPL 64176, an agonist of L-type Ca2+ channels, significantly decreased the axonal mitochondria mobility (17 ± 6%, mean ± SD, p<0.01) relative to that from untreated control neurons (43 ± 10%). In contrast, the same treatment of snph (-/-) neurons failed to affect axonal mitochondria motility (82 ± 8%: before; 73 ± 15%: after the treatment, respectively, p>0.05). This result suggests that Ca2+-dependent and KIF5-Miro-mediated immobilization of axonal mitochondria requires SNPH as an anchor. Second, using GST pull-down and immunoprecipitation of brain homogenates, we identified SNPH as a specific binding partner of KIF5 motor. Furthermore, expressing Miro1 in neurons remarkably increased axonal mitochondria motility (77 ± 14%) compared with the control neurons (33 ± 9%, p<0.01). However, when co-expressing SNPH, Miro1 failed to facilitate mitochondrial transport. Altogether, these results allow us to propose a hypothesis that KIF5-Miro and SNPH share a single system of regulation and this could involve the physical displacement of motor-adaptor proteins by docking interactions (and vice versa). Further analysis of the snph-deficient neurons will provide mechanistic insights into the complex regulation of axonal mitochondrial transport and distribution. (Supported by the NINDS Competitive Fellowship Award and the Intramural Research Program of NINDS)

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Mitochondrial membrane potential is a key indicator of cell health and apoptosis. JC-1, a lipophilic cationic dye, is predominantly used to detect mitochondrial de-polarization due to its selective mitochondrial localization and mitochondrial membrane potential-dependent fluorescence changes. However, the extremely poor water solubility of JC-1 makes it hard to be used for some biological applications. Although JC-1 is widely used with flow cytometry platform, there were few studies that were done with JC-1 using fluorescence microscope or fluorescence microplate
reader platforms due to the low ratio of signal background caused by the JC-1 precipitation in aqueous buffers. A new water-soluble mitochondrial membrane potential indicator JC-10 has been developed to overcome the poor water solubility of JC-1. JC-10 is selectively localized in mitochondria, and its fluorescence change is dependent on mitochondrial membrane potentials. We have used JC-10 to monitor the mitochondrial membrane potential changes in primary rat hepatocytes, CHO-K1, HeLa, Jurkat and HepG2 cells. The mitochondrial membrane potential changes were induced with mitochondrial membrane potential de-couplers and chemicals that induced apoptosis. The consistent EC50 were obtained for mitochondrial membrane potential de-couplers and chemicals that induced apoptosis on three different platforms: flow cytometry, fluorescence plate reader and fluorescence microscopy. JC-10 is characterized as a new robust fluorescent indicator for high throughput analysis of mitochondrial functions in live cells.

702/B649
Effect of the Uncoupler CCCP on the Fatigue of the Slow Fibers of Skeletal Muscle.
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The complex I and III of the mitochondrial respiratory chain are the sites where the superoxide anion is produced, on isolated mitochondria of skeletal muscle (Andreyev et al., 2005). However, mitochondria have a heterogeneous distribution which reflects the metabolic demand, associated with the kind of fiber and muscle (Hood, 2001). The velocity of production of reactive oxygen species (ROS) is related to membrane potential and proton gradient in the internal mitochondrial membrane (Brand et al., 2004). This state is favored in the state 4 of respiration. The carbonylcyanide-3-chlorophenylhydrazone (CCCP) reduced the production of hydrogen peroxide (H2O2) consistent with the fall of the membrane potential characteristic of the transition to state 3 or of a slight uncoupling, confirming that mitochondria is the main source of the production of H2O2 in the muscle. Fibers type I are those that produce less H2O2 (Anderson and Neufer, 2006). We made tension measurements of slow type I fibers of the chick, we realize a dose-response curve (0.5-50 μM). The initial contracture where taken as 100%, then when it fell the contracture to 78.34 ± 1.27 % (fatigued muscle) in maximum and total tension we apply CCCP and we saw an increase to 119.91 ± 41.78 %, (p = 0.028). We consider that the observed effect (in opposition to which we hoped) must be in relation that the application of CCCP eliminates the H2O2 production completely, in the transition to state 3 (slight undocking), which would suggest that mitochondria of skeletal muscle are the main source of the H2O2 production. We could observe as to low concentrations of the uncoupler the tension were increased post-fatigue, which allows us to assume that indeed the ROS production increases the fatigue and that the uncoupler could reduced the ROS production. Acknowledgements: The authors appreciate the partial economic support of the grant of: CIC-UMSNH (2.16, 2009).

703/B650
Loss of MARCH5 Mitochondrial E3 Ubiquitin Ligase Induces Cellular Senescence through Drp1 and Mfn1.
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Mitochondria constantly divide and combine through fission and fusion activities. MARCH5, a mitochondrial E3 ubiquitin ligase, has been identified as a molecule that binds hFis1, Drp1 and Mfn2, key proteins in the control of mitochondrial fission and fusion. However, how these interactions control mitochondrial dynamics and cellular function has remained obscure. Here we show that shRNA-mediated MARCH5 knockdown promoted the accumulation of highly
interconnected and elongated mitochondria. Cells transfected with MARCH5 shRNA or a MARCH5 RING domain mutant displayed cellular enlargement and flattening accompanied by increased senescence-associated-β-galactosidase (SA-β-Gal) activity, indicating that these cells had undergone cellular senescence. Notably, a significant increase in Mfn1 level, but not Mfn2, Drp1 or hFis1 levels, was observed in MARCH5-depleted cells, indicating that Mfn1 is a major ubiquitination substrate. Introduction of Mfn1$^{T109A}$, a GTPase-deficient mutant form of Mfn1, into MARCH5-RNAi cells not only disrupted mitochondrial elongation, but also abolished the increase in SA-β-Gal activity. Moreover, the aberrant mitochondrial phenotypes in MARCH5-RNAi cells were reversed by ectopic expression of Drp1, but not by hFis1, and reversion of the mitochondria morphology in MARCH5-depleted cells was accompanied by a reduction in SA-β-Gal activity. Collectively, our data indicate that the lack of MARCH5 results in mitochondrial elongation, which promotes cellular senescence by blocking Drp1 activity and/or promoting accumulation of Mfn1 at the mitochondria.

704/B651
Differential Distribution of Mitochondria at Nodes of Ranvier Determined by Focused Ion Beam-Based Three Dimensional Electron Microscopy.
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Disrupted mitochondrial function in myelinated axons has been implicated in axonal pathogenesis in diseases affecting myelin but little is known about the factors that regulate mitochondria in myelinated CNS axons in vivo. Recent studies of mitochondrial dynamics in myelinating cultures suggested that axon regions close to nodes of Ranvier have different mitochondrial distributions. To investigate whether this was true In Vivo we compared lengths and volumes of mitochondria in nodal, paranodal, juxtaparanodal and internodal regions of cerebellar white matter axons using three dimensional electron microscopy. We used a focused ion beam "slice and scan" approach using an FEI Nova-200 Nano-lab dual beam system. Resin-embedded tissue was alternately milled using a gallium focused ion beam to remove a 40nm "slice" from the block face and then the milled surface was imaged using a scanning EM in backscatter mode. Automated cycles of milling and imaging produced stacks of 200-400 images representing slices separated by 40nm at x7500 containing sequential slices through 100-200 axons. Axons with nodal regions were traced and volumetric and positional data generated for each mitochondrion. Nodal axoplasm exhibited both reduced numbers and volumes of mitochondria compared with non-nodal regions. Small mitochondria (lowest quartile, <1.6 μm long) appeared uniformly distributed along the axon. Larger mitochondria were rare in the node but abundant in the juxtaparanodal and internodal regions and correlated with stationary mitochondria observed in dynamic studies. Nodal constriction was minor in these small axons, and compensation for changes in axonal diameter did not significantly affect the results. These results demonstrated that myelination can provide topographically differential distribution of mitochondria in the normal CNS axons, and support the hypothesis that mitochondrial dynamics can be focally regulated at the nodes in vivo.

705/B652
Inner Mitochondrial Membrane Protein ChChd3, Is Essential for Cristae Biogenesis and Cellular Homeostasis.
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Mitochondrial cristae membrane serves as the site for ATP production by hosting the respiratory chain super complexes and ATP synthase. Disruption of the cristae structure has been implicated in wide variety of cardiovascular and neurodegenerative diseases. Here, we report that Coiled-coil-Helix-Coiled-coil-Helix Domain containing protein 3 (ChChd3), a previously identified
mitochondrial protein of unknown function, is a critical regulator of cristae biogenesis and oxidative phosphorylation. ChChd3 was originally identified in our laboratory as a PKA substrate in mitochondria, while screening for the potential substrates for PKA in mouse liver mitochondria. Though, ChChd3 was previously known as a mitochondrial protein by global proteomic analysis of mitochondria, so far there is no systematic study describing its localization or the functional role in mitochondria. In our study, we show that ChChd3 is anchored to the IM of mitochondria through its chch domain by associating with the IM protein Mitofilin, which is known to be involved in regulating cristae morphology. Additionally, N-terminal myristylation motif of ChChd3 recruits ChChd3 to the outer membrane (OM) of mitochondria, where it interacts with SAM50, which is known to be involved in the OM beta barrel protein import. Down regulation of ChChd3 in HeLa cells by RNA interference (RNAi) resulted in, major loss of the Mitofilin and Sam50 proteins, accompanied by altered levels in the proteins associated with mitochondrial dynamics and severe defects in the mitochondrial morphology. Mitochondria in cells lacking ChChd3 showed peri-nuclear clumping, fragmentation and loss of cristae followed by drastic decrease in both the cellular respiration and glycolysis, and impaired cellular metabolism. Our results clearly suggest that, ChChd3 is an important contributor to the cristae biogenesis and protein import in the mitochondria, thereby, regulating the mitochondrial function and cellular metabolism.

706/B653

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Thioredoxin reductases are members of flavoprotein family of pyridine nucleotide-disulfide oxidoreductases. These enzymes use NADPH to reduce thioredoxin, a major protein disulfide reductase serving as electron donors for various enzymes including ribonucleotide reductase, peroxiredoxin scavenging H2O2. Therefore, this thioredoxin system is critical for redox regulation of protein function. In this study, we report that two thioredoxin reductases in C. elegans, trxr-1 and trxr-2. GFP reporter system revealed that both trxr-1 and trxr-2 are expressed in intestine as trx-1, a cytoplasmic thioredoxin of C.elegans. Mitotracker staining revealed that trxr-1 was localised in cytoplasm, and trxr-2 in mitochondria as predicted by their signal sequence analysis. It seemed that both trxr-1 and trxr-2 were induced by heat shock and paraquat treatment and less viable under stress condition. Interestingly,acridine orange uptake assay indicated that V-ATPase activity was reduced in trxr-1 and trxr-1;trxr-2 double mutants, while it was comparable to wildtype in trxr-2 mutant. These results suggest that spatial separation of trxr-1 and trxr-2 confer them with differential roles in oxidative stress defense and regulation of enzymatic activity. Oxidized protein levels, transcriptional regulation, and lifespans in different conditions of these mutants are currently under investigation.

Membrane Receptors (707 – 722)

707/B654
Spatial Approximations between Mid-Region of Glucagon-Like Peptide 1 and Its Receptor Provide New Insights into the Molecular Basis of Natural Agonist Binding.
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The glucagon-like peptide 1 (GLP1) receptor is an important drug target within the Family B G protein-coupled receptors (GPCRs). Its natural agonist ligand, GLP1, has incretin-like actions and holds promise for the management of type 2 diabetes mellitus. Despite the recent solution of the structure of the amino terminus of the GLP1 receptor and several close family members, the molecular basis for GLP1 binding to and activation of the intact receptor remains unclear. We previously demonstrated molecular approximations between two carboxyl-terminal residues of GLP1 and the amino terminus of its receptor. In this work, we extended the study to the mid-region of this peptide, synthesizing two photolabile probes that incorporated a benzyoyl
phenylalanine in positions 16 and 20 of the GLP1(7-36) peptide. Both probes bound the GLP1 receptor specifically and with high affinity (GLP1, $K_i=0.7\pm0.1$ nM; position 16 probe, $K_i=2.2\pm0.8$ nM; position 20 probe, $K_i=0.5\pm0.1$ nM). The probes represented fully efficacious agonists, stimulating cAMP accumulation in receptor-bearing-CHO cells in a concentration-dependent manner (GLP1, $EC_{50}=19\pm1$ pM; position 16 probe, $EC_{50}=36\pm8$ pM; position 20 probe, $EC_{50}=37\pm9$ pM). Both probes labeled the receptor specifically and saturably. Peptide mapping by proteinase cleavage of the receptor labeled with the position 16 probe identified the juxtamembranous region of the amino-terminal domain of the GLP1 receptor as the region of labeling, whereas the site of labeling with the position 20 probe was identified as a distinct region of the receptor that includes the second extracellular loop. Radiochemical sequencing will be used to identify the specific receptor residues labeled by each of the probes. Labeling of this region of a Family B GPCR is unique and may provide key spatial approximation to facilitate the meaningful orientation of the amino terminus relative to the helical bundle region of this receptor. This might also help provide insights into its mechanism of activation.

708/B655
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Protease-activated receptor-1 (PAR1) is a G protein-coupled receptor (GPCR) irreversibly proteolytically activated by the coagulant protease thrombin. Thrombin binds and cleaves the N-terminus of PAR1 unmasking a new N-terminus that functions as a tethered ligand by binding intramolecurally to the extracellular surface of the receptor to initiate transmembrane signaling. This unique activation mechanism suggests PAR1 may have distinct modes of signal regulation. N-linked glycosylation is a site-specific enzymatic process, which links saccharides to form glycans that are attached to asparagine (N) residues residing within the consensus sequence NXS/T (X=any amino acid except proline). PAR1 contains five consensus sites: two of which are localized within the second extracellular loop (EC2). To study the effect of EC2 N-linked glycosylation in PAR1 signaling and trafficking, we generated mutants in which the asparagines (N) of the consensus sites were converted to alanines (A). Here, we report that consensus sequences in both the N-terminus and EC2 serve as sites for PAR1 N-linked glycosylation. We found that the PAR1 mutant lacking both N-linked glycosylation modifications at EC2 (NA EC2) trafficked to the cell surface and was cleaved by thrombin like wildtype receptor. However, agonist-triggered PAR1 NA EC2 mutant endocytosis was impaired compared to wildtype receptor, whereas constitutive internalization remained intact. Moreover, activated PAR1 NA EC2 mutant displayed enhanced maximal signaling response compared to wildtype receptor. The effective concentration of thrombin to stimulate half-maximal response after 5 min was lower for PAR1 NA EC2 mutant compared to wildtype receptor. Thus, at face value these findings suggest that thrombin is more efficacious at activating PAR1 NA EC2 mutant than wildtype receptor, suggesting that each activated PAR1 NA EC2 mutant has a greater capacity to couple to signaling effectors than receptors with N-linked glycosylation at EC2. Thus, our studies reveal novel and distinct functions for N-linked glycosylation in regulation of GPCR signaling and trafficking.

709/B656
Identification of a Sphingosine-1-Phosphate Receptor in Drosophila.
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Sphingosine-1-phosphate receptors (S1P1-5) are a subclass of g-protein coupled receptors (GPCRs) that are stimulated by the bioactive lysophospholipid S1P. These receptors have been identified and characterized in various vertebrate models including human, mouse and zebrafish, however, they do not appear to be expressed in invertebrates. In the present studies we have used cell-based functional assays to characterize GPCR-mediated responses to S1P in Drosophila. In two-day old cultures of primary embryonic neurons, both S1P and the specific S1P
analog FTY720 induced rapid neurite retraction in a pertussis toxin (PTX) sensitive manner consistent with GPCR-mediated cytoskeletal rearrangement. In complementary experiments we used transgenic embryonic fly neurons expressing an arrestin2-GFP fusion protein; upon stimulation, the arr2-GFP was predicted to translocate from the cytoplasm to the plasma membrane as is observed in large cultured mammalian cells. Instead, in the relatively small embryonic fly neurons we observed a rapid accumulation of prominent fluorescent puncta along the neurites in response to either S1P or FTY720-P treatment consistent with GPCR activation. In order to identify candidate genes that encode fly S1P receptors, we used a variety of bioinformatic tools as well as microarray data from mutant fly lines in which the accumulation of S1P was genetically altered. Several putative S1P-receptor genes have been cloned into mammalian expression vectors and expressed in HEK293 cells. We are currently characterizing the encoded receptor responses to S1P and its analogs based on stimulation-induced receptor internalization. This characterization of S1P receptors in flies may lead to the subsequent identification of a new class of orthologous receptors in mammals as well as provide insight into their evolution.

710/B657
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The secretin receptor, a prototypic Family B G protein-coupled receptor, forms a constitutive homo-dimeric complex that is stable even in the presence of hormone. Recently, a model of the agonist-bound secretin receptor was built based on high-resolution structures reported for the amino-terminal domains of other members of this family. While this model was fully compatible with all extant data, including ten photoaffinity labeling approximation constraints, a new such constraint now obtained with a position 16 photolabile probe appears to be inconsistent with this model. This has led us to explore whether secretin might dock across both protomers of the homo-dimeric receptor complex. To explore this, we prepared six secretin analogue probes that simultaneously incorporated two photolabile benzoylphenylalanine (Bpa) residues as sites of covalent attachment, in positions known to label distinct receptor domains. These included Bpa\textsuperscript{2,26}, Bpa\textsuperscript{16,21}, Bpa\textsuperscript{16,22}, Bpa\textsuperscript{16,26}, Bpa\textsuperscript{21,26}, and Bpa\textsuperscript{22,26} probes. Each of these dual-reactive probes was shown to represent a full agonist, stimulating cAMP accumulation in secretin receptor-bearing CHO cells in a concentration-dependent manner. Each probe labeled the secretin receptor specifically and saturably, with electrophoretic migration consistent with the labeling of a single protomer of the homo-dimeric secretin receptor. No band representing radiolabeled receptor dimer was observed for any probe except after the control representing covalent cross-linking of the protomers with exogenous disuccinimidyl suberate. The labeled receptor bands were cleaved with cyanogen bromide to demonstrate that both photolabile Bpa moieties had indeed established covalent adducts with a single receptor protomer in the complex. In conclusion, these data are consistent with a model of one molecule of secretin occupying a single secretin receptor molecule within the homo-dimeric receptor complex. This will necessitate developing a model that accommodates all spatial approximation constraints within a single receptor molecule.

711/B658
Palmitoylation of Gp78/Autocrine Motility Factor Receptor (Gp78/AMFR), an Endoplasmic Reticulum-Localized E3 Ubiquitin Ligase.
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Palmitoylation, the covalent association of a lipid palmitate with one or more cysteine residues, is a dynamic post-translational modification important for receptor stability and intracellular trafficking. Using [H\textsuperscript{3}]-palmitate metabolic labeling and anti-Flag immunoprecipitation, we found that full-length and the N-terminal transmembrane domains (N-terminus) of Flag-gp78/AMFR are
both palmitoylated in COS7 cells. Next, we used site-directed mutagenesis to sequentially substitute all cysteines to alanines in the N-terminus of Flag-gp78/AMFR in order to identify the palmitoylation site(s). However, in the absence of all cysteines, the N-terminus Flag-gp78/AMFR construct was still labeled with [H3]-palmitate, suggesting that Flag-gp78/AMFR is undergoing a non-conventional palmitoylation. For most proteins, palmitate is bound to cysteine residues via sulfhydryl bonds, thus we used the Biotin-BMCC assay that reacts specifically with sulfhydryls to form stable thioether bonds to detect cysteine palmitoylation. While full-length gp78/AMFR is palmitoylated on cysteine residues, N-terminus Flag-gp78/AMFR is not, suggesting that N-terminus Flag-gp78/AMFR undergoes unconventional palmitoylation of the N-terminal Flag tag, as previously reported for Sonic Hedgehog (Buglino JA, Resh MD. J Biol Chem. 2008 Aug 8;283(32):22076-88). C-terminal gp78/AMFR cysteines are all located within the RING finger domain, critical to the ubiquitin ligase function of this protein, raising interesting questions as to the relationship between palmitoylation and the ERAD function of gp78/AMFR.

712/B659
Study on Internalization Events and Signaling Compartmentalization of Human GM-CSF Receptors.
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Granulocyte-macrophage colony stimulating factor (GM-CSF) receptor consists of two subunits, α chain and common beta chain (βc). The βc chain is shared by IL-3, IL-5, and GM-CSF receptors, and the β chain is specific to its ligands. Upon GM-CSF binding, GM-CSF receptors transmit signals to modulate cellular proliferation, differentiation, and mobilization. Our laboratory previously found that βc chain recruited an apoptogenic protein, designated as common beta chain associating protein (CBAP), during GM-CSF deprivation and participated in apoptosis modulation. The mechanism by which βc chain distinguishes divergent signaling processes in hemopoietic cells is not well studied. Recently, many studies suggest that internalized receptors upon ligands binding not only were destined for degradation but also had active regulative roles in receptors signaling. Deletion of βc chain C-terminal retained the βc chain on plasma membrane. It indicates the C-terminal of βc chain has an internalization motif for its endocytosis. By serial and internal deleted mutants, we identify that amino acids 474-533 containing a proline-rich motif are responsible for βc chain internalization. The region is also necessary for JAK1 and JAK2 association. JAKs association and phosphorylation is required for activation of βc chain signaling. Some studies reported that JAKs association can target receptor surface expression. However, we found JAKs are dispensable for βc internalization by siRNA knockdown of endogenous JAKs but indispensable for βc signaling. within the region, proline-rich motif is important for both βc internalization and signal activation. Mass analysis of βc associated proteins is processed to identify proteins responsible for βc internalization and signal activation. The mechanism by which internalized GM-CSF receptors α and β chains used to modulate the survival and death signals will be explored.

713/B660
Jak2 Sumoylation and Its Effect on GHR Function.
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Jak2 kinase regulates the signal transduction of many cytokine receptors. Growth hormone receptor (GHR) signalling cascade triggered by GH binding is performed by Jak2, which leads to activation of the STAT pathway and activation of genes involved in growth and metabolism. We found that Jak2 is sumoylated by SUMO2 and SUMO3 up to high molecular weights. Sumoylation of Jak2 is connected with its phosphorylation. Kinase inactive mutants (K882E, Y1007F) have substantially reduced sumoylation signal and constitutively active mutant V617F is hypersumoylated. The significance of that is not yet clear. Currently experiments are being performed in order to identify sumoylation sites, to further investigate how this modification regulates Jak2 function and whether it is a general way of regulating Jak2 family of kinases.
Further experiments will also elucidate whether and how Jak2 sumoylation is connected with GHR signalling and endocytosis.

714/B661
Inactivation of the Proximal NPXY Motif Impairs Early Steps in LRP1 Biosynthesis.
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The proximal NPXY and distal NPXYXXL motifs in the intracellular domain of LRP1 play an important role in regulation of the function of the receptor. The impact of single and double inactivating knock-in mutations of these motifs on receptor maturation, cell surface expression and ligand internalization was analyzed in mutant and control wild-type mice and MEFs. Application of a previously described RMCE strategy for generation of mutant Lrp1 knock-in mice was used to generate a mouse with combined inactivation of the proximal NPXY and distal NPXYXXL motifs in the intracellular domain of the endocytic receptor LRP1. Combined inactivation results in embryonic lethality at the latest at E13.5, reminiscent to the lethal phenotype of Lrp1 knock-out mice. Single inactivation of the proximal NPXY or in combination with inactivation of the distal NPXYXXL motif are both shown to be associated with an impaired maturation and premature proteasomal degradation of full-length LRP1. Therefore only a small mature LRP1 pool is able to reach the cell surface resulting indirectly in severe impairment of ligand internalization. Single inactivation of the NPXYXXL motif revealed normal maturation, but direct impairment of ligand internalization. In conclusion, the proximal NPXY motif proves to be essential for early steps in the LRP1 biosynthesis, whereas NPXYXXL appears more relevant for internalization.

715/B662
Model for Activation-State Dependent Regulation of GPCR Availability.
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We present an experimental and theoretical study about the effects of permanent activation on availability and signaling of G-protein coupled receptors (GPCRs). By directly measuring endocytosis and sub-cellular distribution of a model GPCR, the type-1 cannabinoid receptor (CB1R), we first confirm that activation level, set by constitutive activity and/or chronic pharmacological treatment, and resulting endocytosis are key determinants of steady-state distribution. Kinetic modeling indicates that steady-state activation and relative rates of endocytosis/recycling impose previously unexpected constraints on receptor function. Thus, even at saturating activation levels, active membrane-bound CB1Rs can not exceed approximately 10% of total in HEK293 cells, the remainder forming a dynamic intracellular receptor reserve. Reduced availability is counterbalanced by high-gain coupling to cAMP mobilization (~40 fold), possibly explaining the relatively high efficacy of partial CB1R agonists. The resulting new theoretical framework for GPCR function suggests a spectrum of cell- and GPCR-dependent design trade-offs between maximal sensitivity and robustness. on this spectrum, cells expressing constitutively cycling GPCRs show relatively reduced availability and ligand selectivity counterbalanced by elevated robustness towards desensitization and intrinsic agonist efficacy.

716/B663
The Cell Surface Targeting of Angiotensin II Type 1 and 2 Receptors: Roles of Rab GTPases.
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The angiotensin II (Ang II) type 1 (AT1R) and 2 receptors (AT2R) are the major cell surface mediators for the physiological functions of Ang II. However, the molecular mechanism underlying their targeting to the cell surface remains poorly defined. We found that expression of dominant negative Rab1 mutants (S25N and N124I) and siRNA-mediated depletion of endogenous Rab1 markedly attenuated cell surface expression of both AT1R and AT2R, indicating that their transport to cell surface is mediated through a Rab1-dependent pathway. Consistently, the Rab1 mutants inhibited receptor signaling measured as ERK1/2 activation. Interestingly, expression of wild-type Rab1 augmented the cell surface transport of AT2R, but not AT1R, suggesting that endogenous expression level of Rab1 may differentially modulate the cell surface targeting of AT1R and AT2R. It is of interest to note that expression of wild-type Rab1 increased overall expression of AT2R and its mRNA, whereas the Rab1 mutants attenuated total AT2R expression and enhanced ubiquitin-dependent AT2R degradation. In contrast, expression of Rab1 and its mutants did not alter total expression of AT1R. Furthermore, using similar strategies, we have also demonstrated that Rab2, Rab6 and Rab8 are involved in the regulation of AT1R maturation. These studies demonstrate that multiple Rab GTPases modulate the targeting of nascent AT1R and AT2 to their functional destination (R01GM076167).

**717/B664**
**ARF1 GTPase Modulates A2b-Adrenergic Receptor-Mediating ERK1/2 Activation.**
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ADP-ribosylation factors (ARFs) belong to the superfamily of Ras-related small GTPases and modulate multiple intracellular trafficking processes. Here we report that ARF1 is required for ERK1/2 activation by α2B-adrenergic receptor (α2B-AR). We demonstrated that α2B-AR associated with ARF1 in an agonist-dependent fashion as measured by co-immunoprecipitation. Interestingly, α2B-AR activation upon agonist stimulation also markedly activated the small GTPase ARF1. GST-fusion protein pull down assays combined with progressive deletion and site-directed mutagenesis studies further showed that double Trp residues (diW) located in the third intracellular loop mediated α2B-AR interaction with ARF1. Mutation of the diW motif dramatically disrupted the agonist-dependent association of α2B-AR with ARF1 and attenuated α2B-AR-mediated ERK1/2 activation, without altering the cell surface transport and internalization of α2B-AR. These data demonstrate that the diW motif-directed agonist-dependent α2B-AR interaction with the small GTPase ARF1 modulates ERK1/2 activation by the receptor.

**718/B665**
**Characterization of the Intracellular Trafficking of the Inhibitory Leukocyte-Associated Ig-Like Receptor-1 (LAIR-1).**
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Natural killer (NK) cells, generally recognized as sentinels of the innate immune system, express a variety of activating receptors that are prevented from inappropriately activating cells by co-expressed inhibitory receptors. Little is known about the endocytosis and trafficking of these receptors, which are of great relevance to understanding how NK cells maintain the balance of activating and inhibitory receptors on their cell surface. Our group has recently characterized the unique trafficking pattern of CD94/NKG2A, the ITIM-containing inhibitory receptor expressed by NK and T cells that recognizes HLA-E ligand expressed by most normal cells. We showed that CD94/NKG2A is endocytosed by a previously undescribed macropinocytic-like process that may be related to the maintenance of its surface expression. Here we show that another ITIM containing inhibitory receptor expressed by all leukocytes, the leukocyte-associated Ig-like inhibitory receptor (LAIR)-1, whose ligand are collagens, follow a similar unique endocytic mechanism described for CD94/NKG2A. Our studies suggest that ITIM-bearing receptors share a common bioprocessing pathway that may be related to their function and/or structure, at least in NK cells.
**Role of B Cell Receptor Ubiquitination in Regulating Antigen Processing.**

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B cell receptor (BCR) mediated antigen (Ag) processing and presentation is critical to the initiation of humoral immune response as it leads to B cell - T cell interactions, which support affinity maturation and immunoglobulin class switching. The mechanism of intracellular trafficking of Ag-BCR is not well understood. Our previous studies have established that, in a splenic B cell model, trafficking of Ag-BCR complexes to and within multi-vesicular body (MVB)-like compartments occurs by an ubiquitin-dependent mechanism. It was previously established that B lymphocytes posses at least two distinct endocytic pathways -- clathrin-coated pits and lipid rafts that deliver antigens to distinct intracellular compartments. It was also established that lipid raft endocytic pathway unlike its counterpart was signaling dependent. We hypothesized that ubiquitinated BCR was endocytosed via one of the two distinct endocytic pathways. To further establish the molecular mechanisms behind the ubiquitin-dependent trafficking of Ag-BCR to MVB, we confirmed in another model system, A20uWT (an Ag-specific B cell lymphoma), that BCR was ubiquitinated upon ligand stimulation. Acute treatment of A20uWT cells with proteasome inhibitor MG-132 inhibited BCR ubiquitination and delivery of Ag-BCR to β-hexosaminidase-positive-lysosomal compartment. In addition, treatment of cells with Src Kinase inhibitor PP1, followed by ubiquitin pull-down revealed that, BCR ubiquitination was dependent on Src kinase signaling. Interestingly, by isolating rafts and non-rafts followed by ubiquitin pull-down from each fractions, it was established that ubiquitinated BCR (Ub-BCR) was found predominantly in lipid raft. These results suggest that upon ligand stimulation, BCR ubiquitination is signaling dependent and Ub-BCR is trafficked via the lipid raft dependent endocytic pathway. Future experiments will define the molecular mechanism behind the ubiquitin-dependent trafficking of Ag-BCR to MIIIC.

**Aspirin Regulates Trafficking and Degradation of Membrane Proteins.**

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Acetylsalicylic acid or aspirin is one of the most frequently used drug that acts as an analgesic, anti-pyretic, anti-inflammatory as well as anti-coagulant due to the inhibition of COX (cyclooxygenase). Nevertheless, studies have demonstrated that aspirin, at physiological concentration that was higher than those necessary to inhibit prostaglandin synthesis, profoundly inhibited maturation of dendritic cells (DC), thereby suggesting a COX-independent activity. There was a corresponding decrease in cell surface expression of membrane proteins essential for T cell activation (such as CD40, CD80, CD86, and MHC class II) in DCs upon treatment with aspirin. However, the underlying mechanism remains unknown. Hence, we hypothesized that aspirin regulates trafficking of cell surface membrane proteins in DCs. We used an epidermoid carcinoma cell line, A431, which expresses both Epidermal Growth Factor Receptor (EGFR) and Transferrin Receptor (TfR) as a model in our study to understand the regulation of cell surface membrane proteins by aspirin. We first examined the effect of aspirin on the distribution of EGFR and TfR in A431 cells, and show that aspirin decreases cell surface EGFR. We also demonstrate that aspirin significantly accumulates both EGFR and TfR in A431 cells and that both receptors are trapped in an aspirin-induced compartment (AMC). Further characterization studies show that AMC is EEA-1 and VAMP-3 positive but LAMP-1 negative. In addition, aspirin is shown to have significant effect on the recycling of TfR to cell surface. Thus, the AMC could likely be the early endosomes. Our results suggest that aspirin regulates trafficking of cell surface membrane proteins in mammalian cells, and may shed light on the underlying mechanism that govern the aspirin-induced downregulation of cell surface MHC II molecules in DCs and subsequently inhibition of DC-dependent T cell activation.
Andrographolide (ADO) is the active component of Andrographis paniculata, a plant used in both Indian and Chinese traditional medicine. Studies of ADO on cancer cells have shown that it is capable of inducing apoptosis and cell cycle arrest. However, not much is known about its effect on receptors known to be over-expressed in cancer. In this study, we utilised the well-characterized epidermal growth factor receptor (EGFR) and transferrin receptor (TfR) harbouring epidermoid carcinoma (A-431) cells as a model to study the effect of andrographolide on receptor trafficking. ADO treatment was found to inhibit cell growth, down-regulate EGFR on the cell surface and inhibit the degradation of EGFR and TfR. In addition, EGFR is internalized into the cell at an increased rate, and accumulated in a compartment that co-localizes with the lysosomal associated membrane protein (LAMP-1) in the late endosomes. Our study points towards a new mechanism by which ADO may induce cancer cell death through the down-regulation of cell surface EGFR.

Enhanced VirapowerTM lentiviral vectors provide a highly efficient solution for gene delivery and expression in primary and other cell types which are refractory to more traditional gene delivery methods. Previously we demonstrated optimizations to the VirapowerTM Lentiviral Expression vector line which include new HiPerformTM elements to increase the number of functional viral particles produced (functional titer) and a substantially boost protein expression. Here we demonstrate how these enhancements allow for high levels of transduction efficiency and protein expression in primary neurons and other difficult cell types at high efficiency and low MOI. We also demonstrate the ability of HiPerformTM constructs to deliver and express native, functional membrane proteins including G-protein coupled receptors (GPCRs) in host cells. The ability to produce active GPCRs, capable of antagonist ligand binding and displacement reactions, opens the door to creating novel tools for research, drug discovery and clinical applications.

The localization of Kv2.1 potassium channels into clusters is essential for cellular function, particularly for the determination of cellular excitability and the avoidance of disease. In spite of its clear physiological importance, the mechanism behind Kv2.1 cluster formation and maintenance is largely unknown. Here, we study the dynamics of Kv2.1 channels at the single-molecule level with nanometer precision. Single channels are localized using total internal reflection fluorescence microscopy (TIRFM) with 8 nm accuracy (473 nm laser, 2 mW, 50 ms exposure). Two different fluorophores are used to simultaneously track individual channels and visualize the location of channel clusters. Human embryonic kidney cells and rat hippocampal neurons are induced to express biotinylated Kv2.1 channels fused to green fluorescent protein (GFP) and individual channels are labeled with streptavidin-conjugated red quantum dots (QDs). GFP provides cluster characteristics while QDs enable tracking of individual molecules. Channels
inside GFP-Kv2.1 clusters are imaged in live cells for up to 10 minutes. Clustered channels are mobile with similar diffusion coefficients as that of non-clustered channels (0.03±0.02 μm²/s) but remain confined within the cluster perimeter throughout the imaging time. The mobility of the channels is analyzed by their mean square displacement (MSD) and cumulative distribution function (CDF). Brownian motion is expected to yield a linear MSD (for non-confined channels), and monoexponential CDF. We find that the MSD of non-clustered channel follows a power law instead of linear relation. The CDFs of confined and free channels deviate from the monoexponential form for all trajectories and time lags analyzed (n=900). Tracking single GFP molecules show the same characteristics of QDs, thus we can infer the anomaly effect is not induced by the QD labeling. Our results show that both clustered and non-clustered Kv2.1 channels experience anomalous subdiffusion. The CDF can be fit to a double exponential form that resembles two processes with slow and fast mobility respectively. Our data suggest Kv2.1 channels are mobile in a percolation matrix independent of cluster formation.

724/B671
Long C Terminal Splice Variant Cav2.2 Identified in Presynaptic Membrane by Mass Spectrometric Analysis.
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Cav2.2 voltage gated calcium channels play a key role in the gating of transmitter release at presynaptic terminals. Recently we used mass spectrometry (MS) to analyze the protein complex associated with Cav2.2 in purified presynaptic terminal membranes (Khanna et al., J Biochem Mol Biol 40: 302, 2007). A number of known and new Cav2.2-associated proteins were identified, but not the channel itself. Objective: We set out to explore the above mentioned anomaly and tested the hypothesis that the channel was missed due to high concentrations of co-precipitated proteins. As previously, antibody Ab571 was used to capture the channel from purified synaptosome membrane lysate. Materials and Methods: Cav2.2 specific antibody, Ab571, (Li et al., J Neurosci 24:4074, 2004) was immobilized onto the Protein a agarose gel and incubated with purified synaptosome membrane lysates. Protein complexes captured by the immobilized Ab571 were subjected to a high salt treatment and the remaining proteins were eluted with high concentration urea. Urea eluted protein complexes were analyzed by Liquid chromatography tandem mass spectrometry (LC MS/MS). Results: LC MS/MS analysis of protein complexes that remained bound after the high salt treatment identified 12 distinct Cav2.2 peptides. These peptides were all from intracellular domains of the protein but otherwise their origins span almost the full-length of the protein, including 2 in the N terminus, 4 in the II-III loop, and 6 in the C terminus. One Cav2.2 peptide heralded from the alternatively spliced, long C terminal region. Conclusions: This finding is the first conclusive detection of a presynaptic Cav2-family calcium channel by mass spectrometry and the first detection of this particular channel type in any analysis. Furthermore, one peptide derived from the alternatively spliced, long-C terminal region, confirmed that this Cav2.2 splice variant is targeted to the presynaptic terminal.

725/B672
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INTRODUCTION: CaV3x family channels serve as key conduits for the entry of Ca2+ in numerous processes such as the control of neuronal excitability and muscle contraction. While the ion conductance properties of this channel are well described with high Ba2+ concentrations as the permeant ion little is known about its permeability to Ca2+ at biologically relevant concentrations. Objective: to record single CaV3.x type calcium channel current transients at a external Ca2+ concentrations that span the physiological range. METHODS: Single calcium channels were recorded with the cell-attached version of the patch clamp technique from freshly isolated chick DRG neurons using low noise, quartz electrodes and a cooled-headstage patch clamp system as in1. CaV3.x channels were isolated by electrolyte concentration with ω-
conotoxin GIVA to block CaV2.2, the predominant DRG calcium channel type and 2 μM nifedipine, to block CaV1.x. Channels were evoked using a protocol that first hyperpolarizes to -100 mV to relieve inactivation and then steps from -80 to 0 mV. Channel amplitudes were measured using Clampfit, the Axon suite analysis program by positioning cursors at the closed and open levels by eye. The conductance was determined as the slope through individual channel openings in a current to voltage plot. RESULTS: At 30 mM Ca2+, the single channel conductance was ~6 ps, consistent with previous studies. This decreased monotonically to 1.7±0.1 ps (N=4) at 2 mM Ca2+. CONCLUSIONS: The T type Ca channel exhibits a significantly lower conductance for Ca2+ than the previously analyzed L type channel. This may account for why the other channel types are generally favored for single channel domain-regulated cell signaling processes such as exocytosis. Reference List 1. Church,P.J. & Stanley,E.F. Single L-type calcium channel conductance with physiological levels of calcium in chick ciliary ganglion neurons. J. Physiol 496 59-68 (1996).

726/B673
Regulation of L-Type Ca2+ Channels by Synapse Associated Protein 97.
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Synapse associated Protein 97 (SAP97) is a modular, PDZ domain scaffolding protein important for ion channel targeting and clustering. The α1 subunit of the cardiac L-type (CaV1.2) calcium channel has a C-terminal peptide sequence (VSNL) that interacts with SAP97, however, the functional consequences of this association are unknown. We used a combination of optical and patch clamp techniques to examine the modulatory effects of SAP97 on L-type Ca2+ currents (ICa-L) in HEK293 cells and rat cardiac myocytes. Co-expression of the alpha and beta subunits of CaV1.2 (α1c & β2a) with SAP97 in HEK293 cells resulted in a two-fold increase of peak inward current (ICa,peak) at 0 mV compared to expression of α1c & β2a alone (control). ICa,peak (pA/pF) in control was -9.57 ± 1.37 (n=7) and -20.26 ± 3.93 (n=5) when α1c & β2a were co-expressed with SAP97 (p = 0.015). To test the functional role of SAP97 on ICa-L in native cells, we used a SAP97 adenovirus silencing construct to markedly suppress SAP97 expression in cultured ventricular myocytes (~90% knockdown after 3 days in culture). Whole-cell patch-clamp analysis in ventricular myocytes showed a 60% percent reduction of peak current amplitude. ICa,peak (pA/pF) at 0 mV was -11.24 ± 1.05 (n=6) in control vs. -4.65 ± 0.87 (n=7) following SAP97 silencing (p = 0.0005). Because the targeting of L-Type Ca2+ channels is essential for proper excitation contraction (EC) coupling we next examined the effects of SAP97 silencing on cell Ca2+ transients and contraction. Cells were loaded with a fluorescent calcium indicator (Fura2AM, 2 μM) for simultaneous measurements of intracellular Ca2+ transients and cell shortening (3 Hz, 40 V, 37°C). Ca2+ transients were smaller in SAP97 silenced than in control cells (SAP97 silenced 0.36 ± 0.04 (n = 5) vs. Control: 0.77 ± 0.05 (n = 5, ratio units); p = 0.0001). Cell shortening was also significantly affected in SAP97 silenced cells (SAP97 silenced 0.09 ± 0.01 vs. Control: 0.19 ± 0.03 (µm); p = 0.05). These results show that SAP97 regulates ICa-L targeting probably through PDZ domain interactions. This SAP97 induced regulation may be important in proper EC coupling in ventricular myocytes.

727/B674
RIM1 Regulates Voltage-Gated L-Type Ca2+ Channel Inactivation.
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Insulin secretion by pancreatic β cells involves a tight relationship between Ca2+ influx, via plasma membrane voltage-gated Ca2+ (CaV) channels, and the exocytotic fusion machinery. A molecular array where vesicles are docked in close proximity to CaV channels is necessary for triggering depolarization-induced Ca2+ influx hormone release. Distinct CaV channels have been described based on their biophysical and pharmacological properties. Insulin secretion is coupled to L-type
CaV channel activity, which is activated by strong depolarization and selectively blocked by dihydropiridines. These channels are hetero-oligomeric complexes of a pore-forming (α1) subunit, and auxiliary α2δ and β subunits. RIM1 is a putative effector protein for Rab3, a synaptic vesicle GTP-binding protein, which is a component of the active zone in presynaptic nerve terminals. Recently, it has been described that RIM1 modulates different neuronal CaV channels through interaction with the β subunit, modifying the inactivation rate which translates into a sustained Ca2+ influx and anchoring neurotransmitter-containing vesicles in the vicinity of the channels. Based on these findings, by using a strategy that combines patch clamp recordings with biochemical and molecular biology techniques, we investigated whether RIM1 regulates recombinant L-type CaV channels heterologously expressed in HEK293 cells, as well as native L-channels expressed in insulinoma RIN-m5F cells if this regulation is mediated by the β subunit as occurs in neurons. The most prominent effect of RIM1 on the macroscopic currents was observed on inactivation parameters. Channel inactivation significantly decreased. As expected, RIM1 was unable to affect channel activity in the absence of the β subunit indicating that the physiological association between this protein and the channels occurs via this auxiliary subunit. These results suggest that RIM1 might constitute a functional link between the L-type Ca2+ channels and the machinery for insulin release.

728/B675
CFTR Interactions with the Adenosine 2B Receptor and the β2 Adrenergic Receptor Are Regulated via C-Terminal PDZ Binding.
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CFTR is a chloride channel that plays an important role in regulating airway fluid homeostasis. The β2 adrenergic receptor (β2AR) and the adenosine 2B receptor (A2BR) activate CFTR via G protein linked stimulation of the cAMP/PKA second messenger system. Our study examines the interaction of these two receptors with CFTR at the plasma membrane. In primary human bronchial epithelia cells (HBECs) treated with cytochalasin D (CytD) the ability of the A2BR, but not the β2AR, to stimulate CFTR is lost. In BHK cells expressing CFTR (BHKcftr) A2BR stimulation increased cAMP levels significantly more than in wild-type BHK cells (148±10% vs 24±8% respectively). β2AR stimulated cAMP production was insensitive to CFTR expression. We also confirmed these data in HBECs. Upon CytD treatment, cAMP stimulation in BHK cells was unchanged but in BHK cftr cells the increase in cAMP production was abolished. This effect of CytD was reversed in the BHK cftr cells expressing β2AR, where cAMP increased in CytD treated cells compared to untreated BHK cells (i.e. cAMP with β2AR stimulation was unaffected by CytD).

To determine spatial relationships between A2BR, β2AR, and CFTR we performed FRET analysis. We saw an adenosine (ADO) dose dependent increase (4 to 16%) in FRET between yfpA2BR and cfpCFTR (EC50 = 1.7 μM). Although we determined there was ≈4% FRET between β2AR and CFTR, it was insensitive to isoproterenol (ISO) stimulation. ADO stimulated FRET between A2BR and CFTR was abolished with CytD whilst yfpβ2AR and cfpCFTR FRET was CytD insensitive. We then made A2BR and β2AR constructs that had the c-terminal PDZ binding motifs switched (i.e. A2BRmut1 was switched from type 2 → type 1 and the reverse for the β2ARmut2). In A2BRmut1 cells the FRET response was now insensitive to ADO stimulation. Conversely, by switching PDZ motifs, the β2ARmut2 FRET response was now increased by ISO stimulation. Immunofluorescence in HBECs indicates that actin, ezrin, and NHERF2 were increased in normal compared to CF HBECs. In summary, we demonstrate that A2BR and β2AR interact differently with CFTR. The differences appear to be, at least in part, due to scaffold protein association via the c-terminal PDZ motifs on these receptors.

729/B676
The SK3 (Kca2.3/KCNN3) Channel Is Expressed in Microglia In Vivo And In Vitro And Contributes to Microglial Activation and Neurotoxicity.
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Apamin-sensitive, small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (KCNN/KCa2/SK family), are expressed in the CNS, where they play roles in shaping neuron action potentials. KCa2.3/SK3 is considered a potential therapeutic target for several diseases and genetic disorders involving neuron hyper-excitability. Very little is known about the expression and roles of SK channels in non-neuronal CNS cells or how they are affected by CNS damage. The objectives of this study were to assess: 1) SK3 protein distribution in the rat striatum, before and after transient focal ischemia or intracerebral hemorrhage, and 2) whether the channel contributes to the activation of potentially cytotoxic properties of microglia, the brain’s innate immune cell. We observed extensive KCa2.3/SK3 immunoreactivity throughout the adult rat striatum, and it was especially prevalent in activated microglia/macrophages after stroke. Isolated cultures of rat microglia, neurons and astrocytes expressed transcripts for KCNN1, KCNN2 and KCNN3. Only KCNN3 was up-regulated with microglial activation. We then assessed its role in microglia-mediated neurotoxicity using SK channel blockers in a two-chamber system that restricted drug exposure to microglia (not neurons or astrocytes). Effects of two SK channel blockers were compared: apamin, which blocks SK2 and SK3; and a low concentration of tamapin, which blocks SK2. Our results implicate KCa2.3 channels in microglial activation and their subsequent ability to induce apoptosis of naïve neurons in neuron/astrocyte cultures. Blocking microglial KCa2.3 channels inhibited p38 MAPK activation, and reduced iNOS expression and nitric oxide production, without affecting NF-κB activation or phagocytosis. Consequently, there was less TUNEL, activated caspase 3 and tyrosine nitration in the target neurons. These results implicate KCa2.3 in detrimental aspects of microglial activation, which highlights the need to study functions of non-neuronal cells when considering KCa2.3 as a therapeutic target in the CNS. Supported by grants (LCS) and scholarships (CV, IM-E) from CIHR and HSF Canada.

730/B677
Reduction of TRPM7 Expression Increases Resistance to Apoptosis Stimuli.
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TRPM7, a member of the transient receptor potential (TRP) channel family, is a unique bifunctional protein with kinase and ion channel activities. The channel-kinase is ubiquitously expressed and has been linked to various physiological roles, including magnesium homeostasis, melanopore formation, cell proliferation and cell adhesion. Deletion of TRPM7 in DT40 B cells causes cell cycle arrest followed by cell death. However, a recent study demonstrated that reduction of TRPM7 channel expression in neurons is protective against cell death caused by oxygen glucose deprivation. To better understand the role of TRPM7 in cell proliferation and cell stress we stably reduced TRPM7 expression in HEK-293 cells and Swiss 3T3 fibroblasts by RNA interference. As expected knockdown of TRPM7 in these cells reduced cell proliferation. Interestingly, knock-down of TRPM7 caused the cells to be more resistant to cell stress induced by several apoptotic stimuli, including the application of staurosporine, doxorubicin (adriamycin), and tumor necrosis factor - alpha (TNF-α). TRPM7-knockdown fibroblasts showed reduced apoptosis from these drug treatments as assessed by cleavage of PARP and caspase-3. Re-expression of TRPM7 restored the sensitivity of knockdown cells to apoptotic stimuli; however, TRPM7’s kinase activity was not required, as expression of the kinase-dead mutant TRPM7-G1618D was also successful in reversing the knockdown phenotype. We conclude that depletion of TRPM7 channel from cells enhances their resistance to various forms of apoptotic stimuli.

731/B678
Structural and Mechanical Analysis of Dental Tissues in Mice Lacking the NBCe1 Na+/HCO\textsubscript{3}- Cotransporter.
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Patients with inherited proximal renal tubular acidosis (pRTA) may have extra-renal manifestations including short stature, bone growth delays, and ocular defects. Amelogenesis imperfecta (AI; imperfect enamel) is a group of diverse inherited disorders localized to the dental enamel not usually associated with other symptoms or diseases. The main causes of AI are mutations affecting the secreted enamel-specific proteins, amelogenin, enamelin, kallikrein 4 or matrix metalloproteinase 20. There are however, documented case reports of patients with pRTA who have mutations in the SLC4A4 gene encoding NBCe1 in which dental enamel have also been identified as part of the constellation of extra-renal findings. We recently reported the first evidence that NBCe1 is expressed in polarized rodent dental enamel cells (ameloblasts) at the basal pole. These observations suggest that NBCe1 plays an essential role in enamel formation. Mice with a targeted disruption of the Slc4a4 gene resulting in a total ablation of NBCe1 protein expression could therefore potentially be an excellent model for studying the role of NBCe1 in enamel formation. Here we present analysis of microstructural and mechanical data for the dental hard tissues in Slc4a4/- mice. These animals show hypoplastic enamel with severely disorganized prismatic structure creating an extremely brittle and non-functional tissue. The observed enamel phenotype on the Slc4a4/- animals is similar to AI. Mechanical analysis suggests that the mesenchymally-derived dentine tissue may also be affected although the mechanism responsible for its weaker structure is unknown given that NBCe1 variants are not expressed in dentine-like cells. Taken together these data indicate that abnormal pH homeostasis in developing teeth result in disruption to enamel crystallite formation, creating structurally weak enamel. Normal ameloblast bicarbonate transport/pH regulation is thus required for proper enamel formation.

732/B679
Involvement of a Membrane Transporter, MDR1/ABCB1, in Apoptosis-Independent Sebum Secretion in Hamster Sebocytes.
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Objective: Sebum secretion plays an important role for physiological barrier formation on the skin surface. Although sebum secretion is regulated by a holocrine mechanism that is associated with the apoptosis of sebaceous gland cells (sebocytes), we found a novel insight that sebum secretion is independent of apoptosis in hamster sebocytes. On the other hand, ATP-binding cassette (ABC) transporters participate in the membrane transport of various substrates including lipids. However, whether ABC transporters are associated with sebum secretion is not well understood. In the present study, we examined the involvement of ABC transporters in sebum secretion in hamster sebocytes. Methods: Membrane transport activity and apoptosis-tolerance in differentiated hamster sebocytes (DHS) were monitored by a calcium ionophore, A23187-induced Ca2+ influx, and phosphatidylserine (PS) exposure. ABC transporter activity was measured using an ABC transporter substrate, Rhodamine 123 in the presence or absence of an ABC transporter activator, 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (bzATP). Sebocyte ABC transporter expression was characterized by PCR and Western blotting. Results: DHS that intracellularly accumulated and secreted sebum exhibited constitutive PS exposure without apoptosis. A23187 failed to augment Ca2+ influx and apoptosis in DHS, whereas both reactions were observed with undifferentiated hamster sebocytes (unDHS) that did not accumulate and secrete sebum. Transporter activity was highly detectable in DHS rather than unDHS. BzATP augmented not only transporter activity but also sebum secretion in DHS. PCR and Western blot analyses showed that DHS expressed an ABC transporter—multidrug resistance 1 (MDR1)/ABCB1. Furthermore, an antibody against MDR1 interfered with both ABC transporter activity and sebum secretion in DHS. Conclusions: These results provide novel evidence that sebum secretion is mediated by MDR1 in an apoptosis-independent manner in DHS. Finally, these findings should help accelerate the understanding of the functions of sebaceous glands, and may contribute to the development of therapies for skin diseases such as acne.
733/B680

**Functional Role for Rho-Dependent, Non-Clathrin, Non-Caveolar Endocytosis in Membrane Recovery at the Apical Pole of Bladder Umbrella Cells.**

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Bladder filling stimulates exocytosis of subapical discoidal/fusiform vesicles at the apical surface of the outermost umbrella cells, but the mechanisms by which the added membrane is recovered after voiding and the fate of internalized apical membrane is unknown. Using an In Vitro system that simulates bladder filling/voiding, we observed that apical membrane added during a previous period of filling was rapidly (within 1 min) and completely recovered after experimental voiding. Clathrin/AP2, flotillin, and caveolin-1/-2 were absent from the apical membrane of umbrella cells and inhibitors of clathrin- or caveolar-mediated endocytosis had no effect on apical internalization. In contrast, dynamin-2 was localized along the apical membrane of the cell and apical endocytosis was inhibited by treatment with dynasore or expression of dominant negative dynaminK44A. Further study showed that apical endocytosis occurred in an actin-, Rho-, and Rho kinase-dependent manner, and was triggered by integrins, focal adhesion kinase, and PI-3 kinase. Following endocytosis, membrane bound and/or fluid phase endocytic tracers were initially delivered to ZO1-positive vesicular structures that were closely apposed to the apical junctional complex and were giantin, Rab11a, EEA1, and LAMP2 negative. However, in a subsequent chase the majority of internalized markers were found in LAMP2-positive late endosomes/lysosomes. Our results indicate that during voiding tension stimulates integrin-and possibly FAK/Pi3K-dependent activation of RhoA, which in a dynamin-dependent manner promotes apical endocytosis and subsequent degradation of internalized apical membranes/proteins.

734/B681

**The Role of Sterol Binding of Oxyysterol Binding Protein (OSBP) Homologue ORP1L.**

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Methods to assess the role of sterol binding for ORP1L function we generated a sterol binding deficient mutant (mORP1L) using site-directed mutagenesis. Imaging was performed with confocal microscopy in HeLa cells transfected with tagged DNA constructs. To study binding of [3H] labeled oxysterols, an In Vitro charcoal dextran based assay was used. for cholesterol efflux experiments, foam cell formation was induced by loading RAW264.7 macrophages with [3H]-oleate labeled acetylated LDL. Efflux was determined by liquid scintillation counting. Results Both wt and mutant ORP1L colocalize with Rab7 and Rab7 interacting protein RILP, but not with Rab5, identifying these structures as late endosomes LE. Using live cell and confocal microscopy we show here that sterol binding affects the motility of ORP1L positive late endosomes. ORP1L clusters LEs, mORP1L results in dispersed localization of LEs. Cholesterol efflux is a process where intracellular cholesterol is transported to extracellular acceptor particles. Silencing of ORP1L in acLDL loaded RAW264.7 macrophages was shown to decrease cholesterol efflux to apolipoprotein AI cholesterol acceptor. Overexpression of the mORP1L did not inhibit cholesterol efflux to ApoAI, suggesting that ORP1L sterol binding is not required for the process. To further characterize sterol ligands for ORP1L we show that ORP1L binds 24(S)-hydroxysterol with highest affinity. This is highly interesting considering the fact that both are abundant in the central nervous system. Conclusions In this study we demonstrate that ligand binding affects the positioning and motility of ORP1L positive late endosomes. ORP1L also plays a role in reverse cholesterol transport but sterol binding may not be required for the process. The study supports the role of ORP1L as sterol sensor involved in late endosomal lipid transport.

735/B682

**Activation Mechanisms of the Nucleotide Exchange Factor Arno.**
Upon GDP to GTP exchange, small G proteins of Arf family bind to lipid membranes where they control protein coats, lipid modifying enzymes and other proteins that contribute to the remodeling of cellular membranes. The Arf guanine nucleotide exchange factor ARNO contains three domains: A coiled-coil region, a Sec7 domain and a PH domain flanked by a short polybasic tail. The mechanism by which the Sec7 domain promotes GDP to GTP exchange on Arf is well established but other issues remained to be addressed: which Arf isoform does ARNO activate in the cell and what are the mechanisms that control the membrane recruitment of ARNO? These issues are key for understanding the physiological role of this protein. Recently the group of J. Donaldson has suggested an intriguing cascade of activation (Cohen et al., 2007). Arf6-GTP would recruit ARNO at the plasma membrane, by binding to its PH domain. Thereafter ARNO, through its Sec7 domain would promote nucleotide exchange on Arf1. First, we study this cascade at the molecular level on artificial membranes. For this, we use artificial liposomes containing lipids that are characteristic of the plasma membrane. We observe a strong synergy between phosphatidylserine, PIP2 and Arf6-GTP on the catalytic activity of ARNO on Arf1. These experiments suggest that at least two membrane determinants (PS + Arf6GTP or PS + PIP2) must be present to turn on efficiently ARNO. By examining the activity of mutants of ARNO, we dissect the protein-protein and the protein-lipid interactions that are involved in the Arf6/ARNO/Arf1 cascade. We show that Arf1 activated by this cascade can in turn enhance the activation of other Arf1 molecules by interacting with ARNO in a similar manner as Arf6. We are currently characterizing this cascade of activation in cells, which may contribute to membrane maturation by changing the repertoire of Arf subtypes and specific lipids.

736/B683
Cargo and Adaptor Specific Mechanisms Regulate Clathrin Mediated Endocytosis.
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Diverse cargo molecules (i.e. receptors and ligand/receptor complexes) are taken into the cell by clathrin-mediated endocytosis (CME) utilizing a core machinery consisting of cargo-specific adaptors, clathrin, the GTPase dynamin and numerous endocytic accessory proteins. Previously, we have shown that a subset of accessory proteins, which mediate coat assembly, membrane curvature and cargo selection, can provide input into an endocytic restriction point/checkpoint mechanism that monitors CCP maturation. Furthermore, we showed that overexpression of the constitutively internalized, AP-2-dependent transferrin receptor (TfnR), increases the efficiency of CCP maturation without affecting the rate of CCV budding. Here we report that a different cargo receptor, the low density lipoprotein receptor (LDLR), which uses a distinct set of adaptors (Dab2 and ARH), alters a different aspect of CCP maturation. Using a combination of biochemistry, quantitative live-cell imaging and decomposition of CCP lifetime distributions under various conditions, we show that overexpression of a CD8/LDLR chimera increases the size of CCPs without effecting the efficiency or rate of maturation. This effect is dependent on Dab2 or ARH, but these two adaptors control clathrin polymerization through different mechanisms. We further show that the various adaptors are incorporated into the same CCPs and compete with each other for CCP occupancy. Hence, the ratio of a specific adaptor to clathrin is not fixed but variable. Cargo receptor over-expression also leads to saturation of endocytic efficiency, which we have attributed to saturation of adaptor-mediated cargo sorting into CCPs. Finally, we have identified two additional functions of ARH: as a nuclear protein, exhibiting regulated nucleocytoplasmic shuttling and as a regulator of the actin cytoskeleton. Altogether, our results underscore the highly dynamic and cargo-responsive nature of clathrin coats and illustrate the complexity of CCP maturation. Although it remains unclear what defines the overall CCP density at the plasma membrane, we established that even constitutively internalized cargo and their adaptors can regulate multiple aspects of CCP maturation.

737/B684
Identification of the Switch in Early-To-Late Endosome Transition.
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Sequential transport from early to late endosomes is an important step for protein sorting and degradation that requires the coordinated activities of the small GTPases Rab5 and Rab7. Little is known about the molecular mechanism underlying this transport process. The transition between early and late endosomes could be mediated either through transport carriers or by Rab conversion, a process in which the loss of Rab5 from an endosome occurs concomitant to the acquisition of Rab7. Here we demonstrate that Rab conversion is the mechanism by which proteins pass from early to late endosomes in *C. elegans* coelomocytes. Moreover, we identified SAND-1/Mon1 as the critical switch for Rab conversion in metazoa. SAND-1 serves a dual role in this process. First, it interrupts the positive feedback loop of RAB-5 activation by displacing RABX-5 from endosomal membranes and, second, it times the recruitment of RAB-7 to the same membranes. SAND-1/Mon1 thus acts as a switch by controlling the localization of RAB-5 and RAB-7 GEFs.

738/B685
Isoform Specific Functions of Dynamin 2.
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Dynamin is best studied for its role in clathrin-mediated endocytosis (CME) and most of these studies have employed the first identified isoform, dynamin-1 (Dyn1). However, there are two major, mammalian isoforms of dynamin: the neuronal isoform, Dyn1, and the ubiquitously expressed isoform, Dyn2. It has been assumed that Dyn1 and Dyn2 function redundantly in CME. However, biochemical comparison using a recently developed In Vitro fission assay revealed that Dyn1 but not Dyn2 mediates efficient vesicle formation from planar membranes. However, when membrane tethers, which more closely resemble the highly curved necks of clathrin coated pits are presented as substrates, both isoforms are capable of scission. Consistent with this preference for curved templates, Dyn2 exhibits a much more robust assembly-stimulated GTPase activity in the presence of ~30 nm lipid nanotubes than in the presence of ~100 nm liposomes. These findings point to reduced membrane binding and/or curvature generation by Dyn2. We are currently testing whether interacting partners of dynamin, such as endophilin, amphiphysin or SNX9 that contain BAR domains, which are also involved in curvature generation might enhance Dyn2 activity in these assays. We also recently showed in conditional Dyn2 knock-out cells that CME was more efficiently restored by Dyn2 expression than by Dyn1. Together these In Vivo and In Vitro assays provide a means to identify which domain(s) confer these isoform-specific functional properties through analysis of Dyn1/Dyn2 chimeras.

739/B686
A Switch for Triggering Actin Assembly for Endocytosis.
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Endocytosis in yeast is characterized by an orderly dynamic progression of events at the plasma membrane, starting with marking the site of endocytosis, followed by recruitment of WASp, and then Arp2/3 complex, leading to burst of local F-actin assembly, invagination of the membrane and then scission of an endocytic vesicle into the cell interior. Here we characterize *S. pombe* dip1p, an orthologue of WISH/DIP proteins, which have been proposed to regulate WASp, Arp2/3 and formins in mammalian cells. *dip1* mutants display a defect in endocytosis and possess a marked decrease in the number of active endocytic sites. Time-lapse analysis of actin patch components In Vivo reveal a striking defect in the dynamic progression in the endocytic process. In wildtype cells, the appearance of wsp1p (WASp) at the plasma membrane is followed quickly by actin assembly. In *dip1Δ* cells, wsp1p patches appear normally, but display significant delays (over 10-fold slower on average) in Arp2/3 recruitment, actin assembly and other downstream events. Quantitative kinetic analysis indicate that in the absence of dip1p, rapid activation of the
patch now appears at stochastic intervals, suggesting the presence of a switch mechanism for turning on actin assembly. We provide evidence for positive feedback loops responsible for this switch, which involve actin assembly, WASp and Arp2/3 recruitment, and binding of WASp WH2 domain to the barbed end of actin filaments. These studies thus begin to define a molecular switch triggered by dip1p for the rapid activation of Arp2/3 recruitment and actin assembly at the plasma membrane.

740/B687
Family Ties: Complicated Relationships between the Connecdenn Family of Rab35 GEFs and the Clathrin Machinery.
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Rabs constitute the largest family of monomeric GTPases, yet for the majority of Rabs relatively little is known about their activation and recruitment to vesicle trafficking pathways. Herein we describe the connecdenn family of endocytic proteins. Each connecdenn contains an N-terminal DENN (differentially expressed in neoplastic versus normal cells) domain, a common and evolutionarily ancient protein module. We demonstrate that through their DENN domains, the connecdenns function enzymatically as guanine-nucleotide exchange factors for Rab35. The DENN domain of connecdenn 1 and 2 binds Rab35 whereas connecdenn 3 does not, indicating that Rab35 binding and activation are separable functions of the DENN domain. Through their highly divergent C-termini, each of the connecdenns binds to clathrin and to the clathrin adaptor AP-2. Interestingly, all three connecdenns use different mechanisms to bind AP-2. Characterization of connecdenn 2 reveals binding to the β2-ear of AP-2 on a site that overlaps with that previously described as the interface for autosomal recessive hypercholesteremia protein (ARH) and β-arrestins. However, deletion and mutational analysis reveals that the sequence used by connecdenn 2 is completely unique. The novel connecdenn 2-binding sequence, the K-motif is predictive of AP-2-binding in Rap1-GTP-interacting adaptor molecule (RIAM), which has not been previously linked to clathrin trafficking. Our studies reveal DENN domains as generalized GEFs for Rab35 and identify a new AP-2-binding motif, demonstrating a complex link between the clathrin machinery and Rab35 activation.

741/B688
Structural and Functional Organization of Phosphoinositide and Rab Effectors in the Early Endocytic Pathway.
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Endocytosis is an essential function of eukaryotic cells, providing crucial nutrients and playing key roles in interactions of the plasma membrane with the environment. The classical view of the endocytic pathway, where vesicles from the plasma membrane fuse with a homogenous population of early endosomes from which cargo is sorted, has recently been challenged by the finding of multiple subpopulations of endosomes. These subpopulations vary in their content of phosphatidylinositol 3-phosphate (PI3P) and Rab binding proteins. The role of these endosomal subpopulations is unclear, as is the role of multiple PI3P effectors, which are ubiquitously expressed and highly conserved. One possibility is that the different subpopulations represent stages in the maturation of the endocytic pathway. Alternatively, endosome subpopulations may be specialized for different functions, such as preferential trafficking of specific endocytosed cargo. To determine whether indeed specific receptors are targeted to distinct populations of endosomes, we have built a platform for total internal reflection fluorescence (TIRF) microscopy coupled with structured illumination capabilities. This platform provides the spatial and temporal resolution to distinguish different populations of endosomes and the delivery of fluorescent internalized cargo to these populations in real time. Initial experiments have analyzed the dynamic distribution of two highly conserved Rab5 and PI3P effectors, EEA1 and Rabenosyn-5,
and two internalized cargo molecules, transferrin (Tf) and EGF. Our results indicate that EEA1 and Rabenosyn-5 are found in mostly non-overlapping endosome subpopulations. Rabenosyn-5 containing endosomes are more heterogeneously shaped and on average smaller and more dynamic than EEA1 containing endosomes. at early stages of endocytosis, (<5 min) both Tf and EGF populate Rabenosyn-5 positive endosomal populations, but while Tf remains associated with this population, EGF is transferred to EEA1 enriched endosomes. This approach reveals the detailed structural organization of the early endocytic pathway, and will allow us to determine the specific role(s) of PI3P and Rab effectors in the regulation of endocytic cargo fate.

742/B689
Structural Insights into Dynamin GTP Hydrolysis.
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Dynamin is a large, atypical GTPase that catalyzes membrane fission in the late stages of clathrin-mediated endocytosis. As an unassembled tetramer, dynamin exhibits a high basal rate of GTP hydrolysis that can be stimulated 100-fold by self-assembly on a lipid template. for each of these activities, the underlying mechanism of the hydrolysis reaction remains unknown and the essential catalytic machinery has yet to be identified. Assembly-stimulated activity of the N-terminal GTPase domain can, however, be modulated by mutations in the distal GTPase effector domain (GED). To elucidate the key structural components that facilitate dynamin GTP hydrolysis, we have engineered a minimal GTPase-GED fusion protein (GG) that reconstitutes a robust GTPase activity comparable to full-length dynamin. Using x-ray crystallography, we have solved the structure of GG in the presence of the transition-state analog GDP.AlF4- at 2.0Å resolution. This structure reveals the first high-resolution view of dynamin’s GTPase domain in an activated conformation and suggests a novel mechanism for dynamin stimulation that requires the dimerization of this domain.

743/B690
Sorting Nexin 9 Restricts Dynamin-Catalyzed Membrane Fission to Membranes of High Curvature.
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The large GTPase dynamin is localized to clathrin-coated pits (CCPs) through interactions with multiple endocytic accessory proteins, where it catalyzes membrane fission to release clathrin-coated vesicles (CCVs). Whether such interactions serve as a passive recruitment platform for dynamin or actively modulate the efficiency of dynamin-catalyzed scission of CCPs remains largely unexplored. Sorting nexin 9 (SNX9) belongs to the family of endocytic accessory proteins that can independently bind membranes as well as interact with dynamin and the clathrin-coat components. Using a system of supported bilayers with excess membrane reservoir (SUPER templates), we have previously shown that dynamin alone is sufficient to catalyze fission of both planar and highly curved membranes. We now report that the presence of SNX9 potently inhibits dynamin-catalyzed fission of planar membranes. Interestingly, this inhibition is markedly diminished on highly curved membranes. These results point to a negative regulation of dynamin's function by SNX9 that is dependent on membrane curvature. Such regulation of dynamin's function by an endocytic accessory protein could explain how the growth of a CCP and the resultant increase in membrane curvature at the neck is temporally linked to dynamin-catalyzed scission of the CCP.

744/B691
Mechanism for the Selective Interaction of C-Terminal EH-Domain Proteins with Specific NPF-Containing Partners.
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Eps15 homology (EH) domain proteins can be divided into two classes: those with an N-terminal EH-domain(s), and the C-terminal Eps15 homology domain-containing proteins (EHDs). Whereas many N-terminal EH-domain proteins regulate internalization events, the best characterized C-terminal EHD, EHD1, regulates endocytic recycling. Since EH-domains interact with the tripeptide Asn-Pro-Phe (NPF), the object of our study was to elucidate the molecular mechanisms that allow EHD1 and its paralogs to selectively interact with a subset of the hundreds of NPF-containing proteins expressed in mammalian cells. Here, we capitalize on our findings that C-terminal EH-domains possess highly positively charged interaction surfaces, and that many NPF-containing proteins that interact with C-terminal (but not N-terminal) EH-domains are flanked by acidic residues. Using the recently identified EHD1 interaction partner MICAL-L1 as a model, we have demonstrated that only the first of its two NPF motifs is required for EHD1 binding. As only this first NPF is followed by acidic residues, we have utilized GST-pull-downs, two-hybrid analysis and Surface Plasmon Resonance to demonstrate that the flanking acidic residues 'fine-tune' the binding affinity to EHD1. Indeed, our NMR solution structure complexed with the MICAL-L1 NPFEEEEED peptide allows us to conclude that the first three flanking Glu residues lie in a position favorable for the formation of hydrogen bonds with Lys residues within the EH-domain, and that the second Glu is particularly crucial. Our data provide a novel explanation for the selective interaction of C-terminal EH-domains with specific NPF-containing proteins and allow for the prediction of new interaction partners with C-terminal EHDs.

745/B692
A Single β-Adaptin Contributes to AP1 and AP2 in Dictyostelium discoideum.
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Eukaryotic cells use clathrin coated vesicles to traffic lipids and proteins to specific intracellular locations. The clathrin coat is composed of an outer shell of clathrin triskelions that surrounds an inner shell of clathrin assembly and accessory proteins. Among the most abundant of the assembly proteins are the tetrameric AP2 protein complex, associated with clathrin coated vesicles at the plasma membrane, and the tetrameric AP1 protein complex, found in clathrin coated vesicles associated with the TGN. Here, we describe new contributions of APβ1/2 (β-adaptin) to clathrin function in the soil amoeba Dictyostelium discoideum. We find that, unlike vertebrates, a single β-adaptin subunit functions in both AP1 and AP2 complexes in Dictyostelium cells. Moreover, we find that disruption of the gene encoding β-adaptin results in: (1) mislocalization of clathrin and AP2α, the other large subunit of the AP2 complex; (2) decreased protein levels of mu1 and mu2, the medium-sized subunits for the AP1 and AP2 complexes; (3) cytokinesis defects, and (4) osmoregulatory defects. Similar to β-adaptin, disruption of the gene encoding AP2α results in decreased levels of mu2, but not mu1, suggesting a role for the large subunits of the AP1 and AP2 complexes in stability of their respective mu subunits. Additionally, we find that the cytokinesis and osmoregulatory defects of β-adaptin closely resemble AP1 and clathrin mutants. Finally, differences in the localization of a marker for contractile vacuole membranes in various mutants suggest distinct roles for AP1 in contractile vacuole biogenesis and AP2 in contractile vacuole recycling. Together, our results identify a substantive role for AP1 in clathrin processes, and reveal that β adaptin is indispensable for these functions.

746/B693
Vps45 Modulates Rabenosyn-5 Expression and Regulates Transport at Multiple Trafficking Routes.
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In several invertebrate organisms, the Sec1p/Munc18-like protein Vps45 interacts with the divalent Rab4/Rab5 effector, Rabenosyn-5. In mammalian cells, Vps45 and Rabenosyn-5 also interact, but to date the molecular characterization of this binding, and the functional relationship between these two proteins has not been well defined. Therefore, the objective of this study is to characterize the interaction between Rabenosyn-5 and hVps45 and their functional significance in
vesicular trafficking. Here we identify specific residues within Rabenosyn-5 and Vps45 required for their interaction, by yeast-two hybrid and co-immunoprecipitation assays. We demonstrate that hVps45-depletion leads to decreased expression levels of Rabenosyn-5, likely resulting from Rabenosyn-5 instability and degradation through the proteosomal pathway. Furthermore, we demonstrate that similar to Rabenosyn-5-depletion, hVps45-depletion leads to impaired recycling of β1 integrin receptors, and a subsequent delay in human fibroblast cell migration on fibronectin-coated plates. However, unlike Rabenosyn-5-depletion, which induces Golgi fragmentation and decreased recruitment of sorting nexin retromer subunits to the Golgi, the loss of hVps45 causes Golgi condensation and the accumulation of retromer subunits in the vicinity of the Golgi. Moreover, as a consequence, mannose-6-phosphate receptor retrieval from endosomes to the Golgi is impaired, and the lysosomal hydrolase Cathepsin D displays greater retention at the Golgi. These findings implicate hVps45 and Rabenosyn-5 in post early endosome transport, and we propose that their interaction serves as a nexus to promote bidirectional transport along the endosome to Golgi axis.

747/B694
Potential Role of Pik3c3 in Regulating Neurotrophin Signaling during Sensory Neuron Development.
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The class III PI3K (phosphoinositide 3-kinase) is a member of the PI3K family of lipid kinase. It specifically utilizes phosphatidylinositol as a substrate, producing the single lipid product phosphatidylinositol-3-phosphate (PI3P). While PIK3C3 has been studied in the context of endocytic vesicular trafficking and autophagy in non-neuronal cells, the exact functions of PIK3C3 and its product PI3P in neurons are unclear. We generated Pik3c3flox/flox conditional mutant mice, and crossed them with Wnt1-Cre transgenic mice to delete Pik3c3 in neural crest derived cells including peripheral sensory neurons. Homozygous mutant mice (Wnt1-Cre; Pik3c3flox/flox) are embryonic lethal, dying at E14.5. We found that Pik3c3-deleted dorsal root ganglia (DRG) sensory neurons undergo massive apoptosis beginning at E11.5, despite the fact that they express neurotrophin receptors (Trks) at this stage and all neurotrophins are expressed normally in the peripheral target tissues of DRG neurons. The apoptosis can be rescued when neurons are cultured in vitro, however, cultured neurons have severely retarded axon outgrowth. We hypothesized that in vivo, target-derived neurotrophin signaling, which is essential for the survival of developing sensory neurons, is affected when Pik3c3 is deleted. Indeed we found that pErK1/2 levels are significantly decreased in mutant DRG neurons. Further experiments are being conducted to support the hypothesis.

748/B695
Characterization of Interactions between Clathrin and Its Instrinsically Unstructured Binding Partners.
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AP180 is a clathrin assembly protein containing a globular ENTH domain which interacts with the membrane, and a relatively unstructured C-terminal domain which contains multiple binding sites for clathrin, AP2 and Eps15. Indeed, all known clathrin binding domains (CBDs) are intrinsically unstructured, raising the question of how they interact with clathrin. We characterized how a fragment of the CBD of AP180 (M5), which contains two clathrin binding sites, interacts with the N-terminal domain of the clathrin heavy chain (TD) in solution by NMR spectroscopy. The most significant chemical shift perturbations in AP180 M5 upon clathrin TD binding were observed around the two clathrin binding sites. Results from $^{15}$N T$_2$ and $^1$H-$^{15}$N NOE relaxation measurements of AP180 M5 show that the disordered AP180 M5 polypeptide chain is less flexible at the clathrin binding sites, in both the free and clathrin TD bound states. The $^1$H-$^1$H NOEs of AP180 M5 in both the free and clathrin TD bound states show no NOEs representing alpha helices or beta sheets, indicating that disordered AP180 M5 does not adopt extensive regions of secondary structure upon binding to clathrin TD. However, $d_{NN}(i, i+2)$ NOEs, which are
typically found in beta-turn conformations, are observed between D and F of the DLF motif and between the D and L of the DLL motif, which suggest that M5 has some limited turn-like structure at both clathrin binding sites, whether free or bound to clathrin TD. These repetitive turn-like structures in this flexible and extended polypeptide chain may serve much like hooks on a fishing line, to allow AP180 to recruit clathrin and other accessory proteins from a large volume of cytosol and initiate coated pit formation. (This work was supported by NIH grant NS029051 to EML).

749/B696
Calcineurin Differentially Regulates Dynamin-Dependent Endocytic Processes in C. elegans.
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Objective: C. elegans has been an ideal model organism to reveal In Vivo mechanisms of clathrin-mediated endocytosis. One major clathrin-mediated endocytic process in C. elegans utilizes 6 macrophage-like scavenger cells called coelomocytes to endocytose foreign molecules in the body cavity. Recently, several genes are identified to be involved in coelomocyte endocytosis using C. elegans In Vivo monitoring assay system. However, the detailed mechanism is still unknown. Here, we report a possible function of calcineurin, which is evolutionally conserved a Ca²⁺/calmodulin-dependent Ser/Thr protein phosphatase, in coelomocyte endocytosis. Results: We observed that calcineurin mutants were defective for coelomocyte endocytosis. Genetic data suggest calcineurin functions upstream of the nicotinic acetylcholine receptor CUP-4 to regulate the receptor. Calcineurin also interacts directly with the C. elegans dynamin homolog DYN-1, a large GTPase protein that we show here also regulates CUP-4. Although calcineurin does not appear to be involved in receptor-mediated endocytosis in the oocytes, we observed that both calcineurin and dynamin are necessary for synaptic vesicle recycling in neurons. We speculate that calcineurin may regulate multiple endocytic processes in the nematode by dephosphorylating DYN-1. Conclusions: In this study, we propose a novel working model to control coelomocyte endocytosis in C. elegans. Until now, only a few identified genes involved in coelomocyte endocytosis have been cloned and studied. Therefore, our results showing the identification of a novel regulator and the genetic interaction between novel gene and previously identified genes may help to uncover the exact molecular pathway to regulate coelomocyte endocytosis.

750/B697
The J-Domain and Clathrin Binding Domains Alone Are Sufficient for Gak Activity.
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Cyclin G-associated kinase (GAK), the ubiquitous form of auxilin, is known to be an essential cochaperone for Hsc70 dissociation of clathrin from clathrin-coated vesicles (CCVs). This cochaperone contains a kinase domain, a Pten-like domain, a clathrin-binding domain and a J-domain that binds Hsc70. GAK was knocked out from mouse embryonic fibroblasts (MEFs) derived from GAK-conditional knockout mice by expressing cre recombinase. This caused mislocalization of clathrin adaptors, cargos, and some TGN markers, which was the same phenotype observed in MEFs depleted of clathrin using siRNA, indicating that GAK depletion resulted in loss of clathrin function. While the number of CCPs at the PM and at the TGN was greatly reduced, there was a marked increase in the number of self-assembled clathrin structures devoid of adaptors or cargos in the cytosol of GAK-depleted MEF cells, presumably due to loss of GAK and Hsc70 chaperone function. To test the domains essential for the function of GAK, the
distribution of transferrin receptor (TfR) and cation-independent mannose 6-phosphate receptor (CI-MPR), transferrin (Tf) uptake, and clathrin dynamics were examined in GAK-depleted cells expressing different fragments of GAK. Not only did the full-length and kinase-free GAK give complete rescue, but in addition, there was almost full rescue of TfR and CI-MPR localization, as well as clathrin exchange with a fragment consisting of just the clathrin binding domain and the J-domain of GAK. Therefore, the Pten-like domain, which binds phospholipids, is not essential for recruitment of Hsc70/GAK to CCVs. However, since uncoating is slower without it, it does improve uncoating efficiency. We conclude that the clathrin binding domain along with the J-domain is sufficient for cochaperoning activity of GAK.

New and Emerging Technologies for Cell Biology I (751 – 771)

751/B698
Probing Direct Changes in Live Cell Elasticity in Response to Specific Drugs: a Combined Fluorescence/AFM Study.
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In the present study, we show how Atomic Force Microscopy (AFM) and Inverted Optical Microscopy (IOM) can be used as one single tool to probe in situ direct changes in cell elasticity in response to specific agents. We investigated the effect of two drugs, nocodazole and latrunculin, that are known to enable disruption of tubulin and actin networks respectively. We used MIRO (Microscope Image Registration and Overlay), a powerful automated software allowing fast and easy overlay between optical and AFM images, and a combined AFM-IOM system to target locations on the fluorescence image for AFM force measurements. Elasticity measurements were calculated from the extend part of the approach-retract curves performed in single force or force volume modes, on living Hela cells and two CHO specific cell lines that naturally express fluorescent actin and tubulin, respectively. Results show that nocodazole does not significantly affect cell elasticity whereas latrunculin treatment gives rise to a spectacular change in elasticity indicating that actin cytoskeleton plays a much more important role in cell architecture and mechanics than tubulin network. This work opens the way to exciting outlooks in the field of cancer research.

752/B699
Quantitative and Sensitive Detection of Target DNA Using Nano-Particles as Labels.
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1. Objective Obtaining expression information in a cellular system is essential for understanding the mechanisms of living organisms. The DNA microarray screening has been widely used to obtain such information. The next step in the development of this method is improvement of the detection sensitivity at the single cell level. 2. Methods to improve the detection sensitivity, we tested a method in which gold nano-particles (gold NPs) of several different sizes were to be used as labels. A probe DNA which has a thiol group at the 5’ end was immobilized on surfaces of gold NPs, 10 to 50 nm in diameter. The target DNA was designed so as to be complementary with both probe DNA on the NPs and probe DNA immobilized on a DNA microarray. Hybridization was performed in two steps; first, the target DNA was hybridized with the probe DNA on the microarray, and next the target was reacted with the other probe DNA immobilized on gold NPs. The labeled target DNA was visualized and quantified using a field emission scanning electron microscope (FE-SEM). 3. Results The detection sensitivity was evaluated by using commercially available DNA microarrays with hybridizations of target DNA in the concentration range between 100 aM to 1 μM. Control experiments were performed at other spots on the microarray where non-complementary DNA was immobilized. As results, the detection sensitivity was normally
improved 10 to 100 fold under several different reaction conditions in comparison with conventional fluorescent labeling. The use of NPs of different sizes allowed simultaneous and specific detection of target DNAs having different sequences. 4. Conclusions for assays using DNA microarrays, the use of gold NP labels greatly improved the detection sensitivity toward target DNA. This method is useful for the detection of target molecules at low level expression. on the other hand, relatively long time is required for observation of many spots on a DNA microarray because of the use of an FE-SEM. Therefore, a labeling method should be chosen to suite particular experimental objective. One useful way is reduction of the reaction area on the DNA microarray, which would allow immediate and sensitive detection of target DNA using NP labels.

753/B700
A Novel Fluorimetric Assay for Detection of Calpain Activity with FRET-Based Substrate.
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The calpains are a family of intracellular cysteine proteases. The best-characterized calpains are the isoforms µ- and m-calpain, which are also known as calpain 1 and calpain 2, respectively. Calpains are calcium-dependent and function by modulating the biological activities of many proteins through selective cleavage. Studies have demonstrated that calpains are implicated in a variety of calcium-regulated cellular processes as well as in various pathological phenomena. It has been proposed that calpains represent potential therapeutic targets for drug discovery. Here we report the development of a novel fluorimetric assay for detecting calpain enzyme activity using fluorescence resonance energy transfer (FRET) techniques. We designed and synthesized a new calpain FRET substrate peptide labeled with QXL TM 520 quencher and 5-carboxyfluorescein (5-FAM) fluorophore. Calpain cleaves the FRET substrate into two separate fragments resulting in the release of 5-FAM fluorescence, which can be monitored at excitation/emission= 490 nm/520 nm. Increase in 5-FAM fluorescence is proportional to calpain activity. The assay features a simple "add-mix-measure" protocol and provides a linear range of 0.15 mg/mL- 40.0 mg/mL for calpain 1. The IC50 of two known calpain inhibitors, SJA6017 and B27-WT, is 28.1 nM and 39.6 nM, respectively. The assay can detect both calpain 1 (µ) and 2 (m) activities and is ideal for kinetic study of these enzymes. Moreover, the long wavelength fluorescence of 5-FAM is less interfered by the autofluorescence of components in biological samples and test compounds.

754/B701
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To understand the community effects in cardiomyocyte networks, we developed the on-chip single-cell measurement system combined with on-chip agarose microchamber and multi-electrode array system. The on-chip agarose microchamber system could construct the arbitrary microstructure with photo-thermal etching of agarose layer on the chip using a focused laser beam. The on-chip multi-electrode array system could measure the field potential of cardiomyocytes on the electrode. Firstly, we measured the stability of fluctuation of cardiomyocyte beat rate dependent of cell number using on-chip agarose microchamber system. The beat rate fluctuation tended to decrease as cell-community size increases, irrespective of cell network pattern. Secondly, we observed the response of cardiomyocyte networks to electrical stimulation using on-chip agarose microchamber and electrical stimulation system. The range of stimulation frequency in cardiomyocyte response to electrical stimulation tended to wide as cell-community size increases. Finally, we measured the single-cell level field potential propagation in cardiomyocyte networks using on-chip single-cell measurement system. Contrary to slow conduction velocity of dispersed cardiomyocytes culture, the conduction velocity measured by the field potential propagation in cardiomyocyte narrow linear networks was similar to the action potential propagation in a whole heart. In conclusion, our findings demonstrate that the
community effects in cardiomyocyte networks improve the beat rate stability, response to stimulation, and signal conduction in networks.

755/B702

Angiogenesis and Cancer Cell Invasion in Microfluidic Flow Cells Using Real-Time Microscopy.
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Understanding the molecular and cellular mechanisms of cancer progression from primary malignancy to metastatic disease is critical to development of successful treatments. Cell invasion from the primary tumor and tumor-induced angiogenesis are just two of many phenomena contributing to the pathology of metastatic disease. Both biological processes involve migration and transmigration of cells in response to chemoattractants. In Vitro analysis of these phenomena typically involves deposition of a basement membrane derived matrix (Matrigel) in a transwell plate insert. This allows quantitation of either invasion or angiogenesis, but the inserts can be cumbersome to process. Here, we demonstrate a microfluidic based method to follow angiogenesis or cell invasion in real time. The method enables acquisition of high content data by microscopy using microfluidic flow cells which are optimized for imaging. The channels can be filled with matrix and the device’s fluid exchange capabilities facilitate introduction of cells and compounds. Using this technique, we tested endothelial cell response to bFGF and fumagilin. It was shown that bFGF impregnated in the matrix promoted angiogenesis while fumagilin abrogated the effect. We also investigated invasion of serum starved HT1080 and MCF-7 cells into serum containing matrix and found that as predicted only HT1080 cells successfully invaded the matrix. Using these experimental conditions, the method is amenable to screening many cell-types or many simultaneous conditions or compounds by microscopy in the solid or liquid matrix of a microfluidic channels.

756/B703

Multiphoton Imaging and Nanoprocessing of Stem Cells.
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Objective. Application of multi photon microscopes for safe long-term high-resolution imaging of stem cells during differentiation within 3D microenvironment without the use of exogenous markers as well as for highly-localized nanoprocessing (optical cleaning and targeted transfection). Methods a novel compact nonlinear laser scanning microscope FemtOgen (JenLab GmbH, Germany) with sub-20 femtosecond 75 MHz near infrared laser pulses was employed in addition to a conventional ZEISS LSM510-NLO-Meta system (250 fs at the sample, 80 MHz)). for nanoprocessing, single point illumination of the cell membrane was performed to induce a transient nanopore for the delivery of DNA plasmids vector pEGFP-N1. Animal and human adult pancreatic and salivary gland stem cells were investigated within monolayers as well as spheroids. Results. Near infrared femtosecond laser pulses have been used to realize marker-free two-photon autofluorescence excitation of NAD(P)H and flavoproteins. In addition, second harmonic generation (SHG) was induced within the extracellular matrix structure collagen. Measurements of the same cell spheroids have been performed up to 5 weeks after the incubation with chondrogenic and osteogenic differentiation medium. Differentiated cells exhibited a longer mean fluorescence lifetime and a blue-shifted spectrum. Interestingly, mean powers in the µW range were sufficient to realize non-destructive imaging at GW/cm2 light intensities when using sub-20 fs laser pulses whereas powers of less than 7 mW and low millisecond exposure times were sufficient to realize transient nanoholes at TW/cm2 intensities for transfection. GFP plasmids were optically introduced in stem cells with >70% transfection efficiencies. Furthermore, optical knock-out of single cells of no interest was performed to realize an optical cleaning of stem cell cultures. Conclusion. Ultracompact sub-20 femtosecond laser microscopes may become novel tools for stem cell research. Uchugonova, König, JBO 13(2008)054068 Uchugonova et al. Opt. Exp. 16(2008)9358 Uchugonova et al. J. Biophotonics 1(2008)463
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757/B704
Probing the Role of Cell-Cell Interactions in Tumor Angiogenesis Using Micropatterned Tumor Cell Arrays.
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Microenvironmental conditions contribute to tumor development and progression, however the specific role of cell-cell interactions in tumor angiogenesis remains elusive. To address this question, we have developed a peel-off cell culture array (PeelArray) chip that enables the spatiotemporal control of cell-cell interactions and the unprecedented study of angiogenic factor secretions in response to varying cell-cell interactions. The PeelArray chip consists of a polymer (Parylene) stencil coated on top of a coverslip, which can be easily peeled off to selectively micropattern biomolecules and cells in localized regions. We have used these PeelArrays to create large uniform arrays of fibronectin features of defined surface areas and performed continuous culture of single tumor cells or tumor cell clusters (for OSCC-3 and DU145 cell lines).

A broad assessment of angiogenic factor secretion using cytokine antibody arrays revealed that vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) were differentially expressed by cells devoid of cell-cell contact and cells located within cell clusters. More extensive analysis by immunocytochemistry and ELISA confirmed that cell-cell interactions play a synergistic role in regulating angiogenic potential of tumor cells. Furthermore, inhibitory studies using neutralizing E-cadherin antibodies indicated that the difference in angiogenic factor secretions were related to alterations in E-cadherin signaling. Our PeelArray technology is easy to use and has broad applicability for quantitative biological studies such as mapping protein expression, single-cell behavior studies and studies of microenvironmental effects on stem cells.

758/B705
A Setup for Hypoxia Studies in 24-Well Cell Culture Plates.
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Hypoxic conditions, with the concentration of oxygen reduced below its normal physiological level, have been shown to significantly affect the expression of numerous genes and influence cellular behavior. However, due to the difficulty of generating well-controlled levels of oxygen in cell culture media, studies of cells under hypoxia are typically limited to a single oxygen concentration at a time. To study cells under multiple levels of hypoxia in parallel, we have built and tested a system to impose precise oxygen concentrations, [O2], in cell culture media in 24-well plates. The system uses a novel, computer controlled, multi-channel gas mixer to generate mixtures of N2 and O2 (with 5% CO2 in all mixtures), in which [O2] is set anywhere between 0 and 21% with 0.1% accuracy. Six different mixtures are supplied to six rows of wells of a 24-well plate using flexible strips with micro-machined gas distribution networks and four silicone plugs in each strip (the plugs are inserted into the wells on the plate). In this fashion, we have the ability to study the response of cells to six different levels of [O2] in parallel, with four separate wells for each [O2]. As a first test of the system, we have measured the mortality rate of mammalian cells exposed to different levels of [O2]. We have found significant differences between cells under hypoxia ([O2] < 4%) and cells in media saturated with air ([O2] = 21%). Specifically, during a period of 15 hours, the mortality rate of hypoxia-treated mouse cortical neurons was a factor of 2 higher than for neurons under control conditions (0.96% dead/hr and 0.52% dead/hr, respectively). In addition, we have used the system with cultures of mouse cortical neurons, fibroblasts, and glial cells to quickly determine the threshold of hypoxic response in different cell lines as well as to study the effect of hypoxia on gene expression. A specific advantage of our system is that standard 24-well plates can be adapted to quantitative studies of hypoxia with cellular responses to six precisely defined oxygen concentrations tested in parallel.

759/B706
High-Throughput Assessment of Cellular Mechanics and Underlying Physiologies.
Cellular mechanics regulates various physiological functions of tissues and organs including their integrity and development. Disturbance of this regulation can result in fatal diseases such as fibrosis and atherosclerosis. To understand how cell mechanics affects tissue functions or vice versa, we developed a three dimensional hydrogel tissue (HT) assay system that simultaneously quantifies the contractility and multiple physiological parameters of cells. Rat embryo fibroblasts (REF52) suspended in a collagen matrix was incubated to form HT membranes around scaffolds in wells that conform to 96-well plate format. To assay cellular mechanics, we used a robotic device, Palpator™, that can automatically measure the mechanical properties of eight HTs in 5 min. To assay cellular physiology, F-actin content and mitochondrial potential (MMP) were monitored using the fluorescent markers Alexa™-conjugated phalloidin and tetramethylrhodamine, ethyl ester. We also assessed cellular viability using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium. To demonstrate the application of this assay system, we successfully screened for a small molecule that modulates contractility without affecting actin integrity, mitochondria activities or cell viability. Four test compounds were assayed: Rho kinase inhibitor (H1152), cytochalasin D (CD), 2,4-dinitrophenol (DNP), and rotenone (ROT). DNP, CD, and H1152 treatments (3 hr) dose-dependently reduced HT contractility with EC50 of 1300, 0.2, and 0.1 μM respectively. Treating HT with DNP dissipated MMP with EC50 of 340 μM. CD treatment (24hr) reduced F-actin labeling and viability to 75% of non-treated control. Although ROT did not affect contractility, it significantly reduced viability (55% of control). While all four compounds reduced HT force, we found H1152 to have the least negative effects on cellular physiology. To better characterize HT physiology, we are developing assays to measure ATP concentration, O2 metabolism and free radical production. Our HT-based assay system would be useful for revealing underline mechanisms that regulate cell and tissue mechanics. Further, the system can be used for high-throughput compounds screening.

760/B707

Development of a Novel Screening System for Claudin Binder Using Baculovirus Display.

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Objective: Claudin (CL), a tetra-transmembrane protein, is a component of tight junction (TJ) in epithelium. CL comprises a family containing 24 members. A CL-4 binder (C-CPE) is a potent mucosal absorption enhancer. Most of malignant tumors are derived from epithelium, and overexpression of CL is frequently observed in the tumors. These findings indicate that CL binder is a promising molecule for pharmaceutical therapy. However, because of the hydrophobicity, it is difficult to prepare CL protein in an intact form on the plate. There is little success of developing CL binder by high-throughput screenings. Recently, a novel type of protein expression system using baculovirus has been developed. Membrane proteins are displayed on the budded baculovirus (BV) in their active form (Sakihama et al., 2008). In the present study, we developed a method to screen for CL binders by using BV display system. Methods: CL-displaying BV (CL-BV) was prepared as described previously (Saeki et al., in press). Interaction of CL-BV with C-CPE was evaluated by enzyme-linked immunoassorbent assay. C-CPE-displaying phage (C-CPE-phage) library was prepared by random mutation in the functional amino acids. Modulation of TJ-barrier was evaluated in Caco-2 monolayer cell sheets. Results: C-CPE bound to CL-4-BV but not to mock-BV. In order to apply the BV display system to screening for CL-4 binders, we constructed a C-CPE-phage, which interacted with CL-4-BV. C-CPE-phages could be successfully selected from a mixture of C-CPE-phage and scFv-phage by using CL-4-BV. Moreover, novel CL-4 binders were also screened in C-CPE phage library. The C-CPE derivatives modulated TJ-barrier. Conclusion: The CL-displaying BV system may be a promising method to produce a novel CL binder and modulator.
761/B708
Iporation: A Novel Technology for High-Throughput Sirna Delivery in Primary and Hard-To-Transfect Cells.
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Effective siRNA delivery in cultured cells has great utility in basic research as well as therapeutic development. Primary cells directly derived from tissues represent superior In Vitro models mimicking In Vivo physiology and pathophysiology. Traditional transfection methods, such as lipofection and electroporation, have limited utility in delivery siRNA in delicate primary cells due to low efficiency and high cellular damage. Also, the chemical reagents used in lipofection and electroporation buffers often cause off-target effects on many genes, obscuring the desired gene silencing effects from specific siRNAs. We have developed a novel iPoration technology in 96/384-well formats to address the unmet needs in high-throughput siRNA delivery in primary cells and hard-to-transfect cell lines. Unlike traditional electroporation that uses high-voltage pulses to treat cells in suspension, iPoration injects precise electrical currents into adherent cells grown on semi-porous membranes, thus induces temporary membrane permeation in the cells. This novel approach consistently produces over 90% siRNA delivery efficiency with virtually no cellular damage in various cell types including differentiated primary endothelial and epithelial cells, stem/precursor cells, and hard-to-transfect cell lines such as Caco-2. Using the iPoration technology, we routinely achieve over 75% gene knockdown effectiveness at siRNA concentrations of 100nM or less, twenty times less than what is needed for a currently available electroporation-based method. In a genome-wide microarray analysis of primary Human Cardiac Microvascular Endothelial Cells (HMVEC-C) treated with iPoration, non-specific effects (log2 ratio >1) are seen only in 0.34% of the nearly 31,000 genes analyzed, which is more than 10-fold less compared to previously reported data from lipofection. These results demonstrate that our novel iPoration technology is an ideal tool for high-throughput siRNA delivery in primary and hard-to-transfect cells, and will have great applications in functional genomics, target identification and validation, and siRNA therapeutic development.

762/B709
PSI: Biology High-Throughput Enabled Structural Biology.
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The primary goal of the Protein Structure Initiative:Biology (PSI:Biology) initiative to be funded by the NIGMS is to apply high-throughput structural biology to important biological problems. This will be accomplished by establishing partnerships between centers for structure determination and biologists with interests in particular proteins or collections of proteins. The PSI:Biology network centers will include: 1) Centers for High-Throughput Structure Determination, 2) Centers for Membrane Protein Structure Determination, 3) the PSI:Materials Repository, and 4) the PSI:Biology Knowledgebase. The partnerships, established through Consortia for High-Throughput-Enabled Structural Biology Partnerships, will define targets for structure determination and provide funds for functional studies in the applicants' laboratories and for a portion of the cost for structure determination in the center. In addition to protein structures and models, the PSI:Biology network will generate and make available reagents and plasmids for expressed proteins to support functional studies in the research community. NIGMS encourages Partnership applications from biologists or groups of biologists with biological questions that will benefit from the determination of relevant protein structures. The PSI:Biology high-throughput approach will enable examination of families of proteins related to the target proteins, an approach that has proven highly successful in generating the first structure of a family member and then allowing many other family members to be modeled. Examples of current partnerships include using structural genomics, modeling and systems biology to generate a three-dimensional reconstruction of the central metabolic network of the bacterium, Thermotoga maritima, and the discovery of novel enzymatic mechanisms for the enoylase and amido hydrolase classes of enzymes. Additional opportunities for researchers to join the PSI:Biology network will be provided.
through ongoing and future program announcements. Researchers may also suggest proteins for structure determination through the PSI Community Nomination site at: http://cnt.psi-structuralgenomics.org/CNT/targetlogin.jsp.

763/B710
Design and Validation of a Multiforce High Throughput System (MHTS) for Measuring Single-Cell Mechanics.
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We report on a Multiforce High Throughput System (MHTS) for high content screening (HCS) of cell mechanics. In the past decade, HCS has changed the way assays, such as binding and motility, are performed. Independently, there is increasing understanding of the role of mechanics in cell sensing, signaling, and differentiation. However, there has been little intersection between these two trends: low throughput techniques dominate mechanical study of biological systems. We present a system that applies forces to microspheres using a magnetic field gradient. The spheres can be adhered to cell membranes to measure cell mechanics or elicit a biochemical response. The MHTS has a wide force range of 1pN - 10 nN, depending on particle size. The system temperature can be controlled in the range 20 - 40°C, and MHTS microplates have been used to grow cell cultures. The MHTS is compatible with high-NA fluorescence microscopy. We have engineered novel magnetic field geometries that increases the usable microplate area for a cell mechanics experiments. We have used the MHTS to explore the role of PTPase, an enzyme known to be involved in force sensing, on the compliance of Drosophila fly cells. Spring constants were $760\pm70$ N/m and $240\pm50$ N/m for normal and PTPase-inhibited cells, respectively. These initial studies show promise for mechanical screening of the RNAi library available for these cells. Finally, we report on our latest results applying the MHTS to measure metastatic potential of cancer cells.

764/B711
The Split-TRP System: A Versatile Protein Screening Platform for the Identification of Protein-Protein Interactions in All Cellular Compartments.
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Yeast two-hybrid based systems are powerful tools for the detection and characterization of protein-protein interactions (PPIs). However, some important protein classes, e.g. integral membrane proteins and transcription factors are difficult to investigate using two-hybrid technologies. To overcome these limitations we employed a novel protein complementation screening platform. PPIs are detected by reconstitution of the split-protein sensor TRP1, enabling trp1 cells to grow on medium lacking tryptophan. Since the interaction readout is direct and independent of transcriptional reporter activation the rate of false positives is lowered considerably. Furthermore, the technology allows detection of PPIs in their natural setting, e.g. the cytosol, the nucleus or at cellular or organellar membranes. Several proteins involved in human diseases, including the cell cycle checkpoint protein p53, the amyloid precursor protein APP or the ion channels cystic fibrosis transmembrane conductance regulator and betaENaC were successfully tested in pairwise interaction studies, demonstrating the universal applicability of the system. Screening p53 and APP against human cDNA libraries validated for the isolation of novel binding partners from cDNA expression libraries. This project was funded in part by the European Union Framework 6 Grant “Target Screen 2”, contract number 037365.

765/B712
Cellular Signaling in Tnfα Stimulated A549 Lung Carcinoma Cells: Phospho-Protein and Cytokine/Chemokine Multiplex Profiling.
Signaling events initiated by extracellular receptor activation lead to the expression and release of cytokines/chemokines. These events are central to cellular and tissue homeostasis, development and functional response. The complexity and number of protein targets involved in such signaling events and cellular responses that cells may undergo require tools that enable multiplex analysis of samples. Here we demonstrate the use of Luminex® technology for the multiplexed analysis of cell signaling and cytokine/chemokine expression following TNFα stimulation of A549 cells. Tissue culture supernatants and cell lysate samples were collected over a 24 hour time course from TNFα stimulated cells. These samples were analyzed using MILLIPLEX® MAP and MILLIPLEX® EpiQuant immunoassays, respectively. Of the 42 cytokines/chemokines investigated, an increased expression level (as compared to vehicle control) was observed for 21 targets in cell culture supernatants. Of the 78 phospho-tyrosine cell signaling targets analyzed, a response was observed in 43 target sites. The magnitude of cytokine/chemokine and phospho-tyrosine response varied significantly between targets. for several phosphorylation sites, a decrease in phosphorylation was observed in response to TNFα treatment. Results were obtained using fewer than 288 microplate wells (three-96 well plates), significantly reducing sample and reagent consumption and laboratory time. This approach clearly demonstrates the applicability and utility of coupling intracellular multiplex screening with the measurement of secreted factors - the end-product of many signaling pathways.

766/B713
The PSI Structural Genomics Knowledgebase - A One-Stop Shop for Protein Structure, Function, Sequence, Methods, and More.
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To enable biological research, the Protein Structure Initiative Structural Genomics Knowledgebase (PSI SGKB) integrates biological and structural data for all protein sequences. Researchers can use the PSI SGKB to jump-start new biological projects or find solutions to laboratory bottlenecks. With a text, PDB ID, or sequence, users can find matching PSI and PDB structures; view related annotations about sequence, structure, and function; interact with computational 3D models; find protein production protocols; purchase available DNA clones and vectors; and discover new technologies developed by the PSI for protein production, structure determination, and analysis. In collaboration with the Nature Publishing Group, the PSI SGKB provides a research library, editorial about new research advances and technologies, news, RSS feeds, and an events calendar to present a broader view of structural biology and structural genomics. A description of how this resource can be used for research will be presented. The PSI SGKB is funded by the NIGMS.

767/B714
The NCI-Nature Pathway Interaction Database (PID, http://pid.nci.nih.gov; Schaefer et al., 2009) is a freely available collection of professionally curated and expert-reviewed signaling and regulatory pathways composed of human molecular interactions, signaling events and cellular processes extracted from primary literature. Pathways selected for curation are based on potential drug targets, suggestions made by our users and reviewers, and other cell signaling molecules. As of September 2009, the database contains 97 pathways encompassing 5650 interactions or events, 2794 proteins, 138 small molecules, 2219 complexes and 2152 peer-reviewed publications. Created in a collaboration between the U.S. National Cancer Institute and Nature Publishing Group, the PID is aimed at researchers interested in cell signaling pathways, such as molecular cell biologists, and bioinformaticians. The database offers a range of tools to facilitate pathway exploration. Users can browse the pre-defined set of pathways and create network maps centered on a single molecule or biological process of interest. The Batch query tool allows users to upload molecule lists, such as those derived from microarray data, and visualize the resulting molecular connectivity map. In addition, users can download lists of proteins, references used to create the pathway and complete database content in extensible markup language (XML) or Biological Pathways Exchange (BioPAX) format. The database is updated every month and supplemented by a concise editorial section that provides synopses of recent noteworthy papers in cell signaling and specially commissioned articles on the practical uses of other relevant Bioinformatics tools. Users can sign up for email alerts or RSS feeds to receive database updates.

768/B715

Automated Visualization of Subcellular Environments.
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The elaborate biological machinery that allows our cells to function properly, or that can cause disease when broken, exists on a scale that is too small to see with a microscope and too large to visualize with nano-inference technologies like x-ray crystallography. This fact makes the fundamental causes of both health and disease difficult to study as it forces expert and student alike to abstract a mental picture of life’s most complex spatiotemporal interactions from countless data that spread across dozens of disciplines. Until science invents more direct methods of observing the mesoscale, we can, at best (and at least), assemble nanoscale structures onto microscale data using distribution functions gleaned from other branches of biology with varying reliability. In an effort to better comprehend cell function, we have begun to contribute a visualization standard that can accept, manipulate, and even assess the data produced across multiple scientific disciplines- to frame the details of molecular biology on a broader cellular map and to extrapolate predictions where reasonable. A computational toolkit we develop aims to provide ease and accessibility to mesoscale content to both the scientific community, by allowing for more efficient and non-redundant hypothesis driven research, and to educators and learners of all levels via the selective filtering and post-processing of complex subcellular data. Our toolkit's algorithm models molecular elements into cellular electron tomograms automatically. By directly accessing raw data inputs, the software can deposit molecular detail across surfaces and throughout volumes to simulate cellular membranes and compartments. Here we show examples of the algorithm in action as it fills both prokaryotic and human whole-cell tomograms with molecular detail. We show options for interactively conveying confidence across the different parameters in the model and explain how ports reaching out to various databases can continuously provide input to each modeling algorithm to alter these confidence values. Finally, we show immediate applications for education and outreach while the algorithms continue to develop towards research standards.

769/B716

RandTag: Proteome-Wide Study of Protein Subcellular Location in NIH 3T3 Cells.
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The RandTag project is a collaborative effort to image the subcellular distributions of fluorescently tagged proteins in NIH 3T3 cells. The project goal is to obtain a proteome-wide description of the subcellular localization of proteins in this mammalian cell line. CD-tagging is used to obtain clonal cell lines in which a new exon containing eGFP is inserted into the chromosomal copy of a random gene. Tens of thousands of wide-field images of these cell lines are generated each week using automated fluorescence microscopy. Some clones are also selected for confocal imaging using a computer-assisted decision making process. The location of each GFP guest exon is determined by DNA sequencing; and literature-based information about each tagged protein is also collected. For handling this large quantity of heterogeneous data, we use automated, machine-learning based, computational methods. In particular, we employ algorithms previously developed and validated by our group for the image-processing pipeline. We have also developed a graphical model to integrate the various sources of information, taking into account their respective importance (e.g., a literature reference on the same organism is more relevant than one based on homology). Release 1 of information on over 3,000 clones has already been made publicly available through our Protein Subcellular Location Image Database, and new releases are planned every six months. The project is on going, with over one hundred clones being analyzed each week. An upcoming release will contain not only images for all proteins but generative models of subcellular patterns that can be used in systems biology simulations.

770/B717
A Comprehensive Bioinformatics Analysis of HDAC-Interacting Proteins.
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Histone deacetylases (HDACs) are enzymes that regulate the functions of histone and non-histone proteins by catalyzing the removal of acetyl groups from lysine residues. They control gene expression by altering chromatin organization and dynamics. In addition, HDACs function as key regulators of many cell processes including intracellular trafficking, autophagy and organelle turnover, ciliary disassembly, cell matrix interactions and signaling, cell migration, cell death, and cell-cell Interactions. Many proteins that have previously been described as protein modification enzymes represent only single members of protein complexes and only function properly together with other factors. Increasing evidence suggests that HDACs may also require additional factors for their various functions. Using publicly available databases and bioinformatics tools such as BLAST, we were able to identify putative interacting proteins of eighteen human HDACs by constructing a “round trip” between human HDACs and their interacting proteins through homologues in other species that are experimentally verified to interact. A database was developed by combining many data sources containing protein-protein interaction information, and a fully automatic method was developed to query, annotate and identify potential interacting proteins, which can be applied to any protein of interest. Our findings that HDACs associate with a diverse number of different proteins lead to many questions. For example, do proteins that interact with HDACs stimulate or antagonize HDAC activities? Do HDACs reciprocally alter the functions of these HDAC-binding proteins? Work is now underway to further analyze the proteins that associate with HDACs and to determine how HDACs modulate the functions of these proteins. Knowledge of these interactions will provide a basic understanding of the mechanism of each HDAC and add to our understanding of how HDACs regulate many important biological processes.

771/B718
The Rational Design of Predicted Neuraminidase Inhibitors for the Treatment of Swine and Avian Influenza.
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The World Health Organization warns about the pandemic potential of the H5N1 and H1N1 strains of influenza because of developing drug resistance and human-to-human transmission. In the current study, virtual docking of fragments derived from FDA approved drugs followed by fragment linking within the neuraminidase (N1) enzymatic pocket is used to increase the library of available molecules for virtual screening. Though virtual screening is a well-established technique, it does not typically account for the entire range of receptor flexibility. The Relaxed Complex Scheme (RCS) partially resolves this problem by integrating data from molecular dynamics simulations with computational ligand-receptor docking to get an accurate ensemble binding energy average for each ligand. Here, we generated fifteen novel molecules with better-predicted binding energies than all known neuraminidase inhibitors like oseltamivir, peramivir, and zanamivir. Additionally, our preliminary analysis suggests that these inhibitors have a binding mode distinct from that of oseltamivir and zanamivir. They seem to bind farther from mutations known to confer resistance. Future studies include expanding the list of potential candidate molecules followed by experimentally validating our new library in their ability to inhibit these viruses. The wider implication of this study is to increase the probability of identifying drugs with the ability to fight rare and lethal strains of influenza, especially those with resistance to known drugs.

**Cell Culture (722 – 794)**

**772/B719**

**Purification and Characterization of a Monocot Lectin Having Anti-Proliferative Effect on Human Cancer Cell Lines.**

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Objective: Lectins are defined as carbohydrate binding proteins other than enzymes and antibodies. Lectins have emerged as very important macromolecular tools to recognize carbohydrates on cell surfaces. The present work is designed to purify and characterize monocot lectins with interesting biological properties from Indian monocot plants. Methods: on the basis of sugar specificity determined by hemagglutination, asialofetuin-linked affinity was used to purify monocot lectins. Anti-proliferative potential was determined with sulphorhodamine-B assay. Results: Arisaema utile lectin (AUL) gave a single band in SDS-PAGE at pH 8.3 corresponding to subunit Mr 13.5 kDa. The native molecular mass of 54 kDa suggested a homotetrameric structure. Like other monocot lectins, AUL gave multiple bands in isoelectric focusing and in native PAGE at pH 8.3. AUL was inhibited by N-acetyl-D-lactosamine (LacNAc), a disaccharide and asialofetuin, a complex desialylated serum glycoprotein. When treated with denaturing agents, the lectin was stable in the presence of urea (3 M), thiourea (4 M) and guanidine HCl (4 M). The lectin had no requirement for divalent metal ions i.e. Ca2+ and Mn2+ for its activity. Amino acid analysis revealed high content of aspartic acid, glutamic acid, glycine and threonine and a very low amount of methionine but complete absence of cysteine. Amino acid modification studies of AUL revealed the involvement of tryptophan and tyrosine residues involved in lectin-sugar interaction. Using Far UV CD spectra the estimated secondary structure was 37% α-helix, 25% β-sheet and 38% random contributions. In Vitro anti-proliferative activity of AUL was tested on eleven different human cancer cell lines viz. MCF-7 (Breast), SK-N-SH (CNS), 502713 (Colon), Colo-205 (Colon), HCT-15 (Colon), HT-29 (Colon), SW-620 (Colon), Hep-2 (Liver), IMR-32 (Neuroblastoma), DU-145 (Prostate) and PC-3 (Prostate). The concentrations of AUL which produced 50% inhibition (IC50) of cancer cell lines viz. SW-620, HCT-15, SK-N-SH, IMR-32, Colo-205 and HT-29 at 38, 42, 43, 49, 50 and 89 µg/ml, respectively. Conclusion: The purified Arisaema utile lectin was found anti-proliferative towards human cancer cell lines.

**773/B720**

**Actin Cytoskeleton and Extracellular Matrix Remodeling during Early Adipocyte Differentiation.**
Adipocyte differentiation at an early stage is directly regulated by gene expression of a master regulator such as peroxisome proliferator-activated receptor-γ2 (PPARγ2). During adipogenesis, extracellular matrix (ECM) remodeling defines important events of the differentiation process. This is characterized by conversion from the fibronectin (FN)-rich stromal matrix of a preadipocyte to the basement membrane of an adipocyte. Adipocyte differentiation has been reported to result in the conversion of filamentous actin from stress fibers and lamellipodia to cortical actin structures. However, the order of the following events has not been clarified: (a) PPARγ2 expression, (b) ECM remodeling, and (c) actin cytoskeleton remodeling. In this study, we aimed to elucidate the remodeling of the actin cytoskeleton and ECM during early adipocyte differentiation. Before adipogenic induction, preadipocytes showed fibroblastic morphology with a well-developed actin stress fiber pattern and formed FN networks in connection with their actin cytoskeletons. After differentiation for 24 h, the actin fibers were disassembled and disorganized, and as a result, the FN networks were also disorganized. After 48 h of differentiation, the fibers disappeared and a cortical actin structure was formed. Coincidentally, the FN networks degraded and disappeared. After 60 h of differentiation, lipid droplets accumulated in the cytoplasm and laminin-8 expression was detected on the plasma membrane. Matrix metalloproteinase (MMP)-2, a catabolic enzyme essential for ECM remodeling, was activated after 48 h of adipocyte differentiation, whereas MMP-9 was not detected. Furthermore, PPARγ2 increased significantly after 48 h of differentiation. These results indicate that remodeling of the actin cytoskeleton and ECM precedes PPARγ2 expression during adipocyte differentiation, and therefore, we suggest that this remodeling acts as a trigger for adipocyte differentiation.

774/B721
The Nanomechanical Signature of Tumorigenic Transformation.
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Cells within tissues continuously encounter dynamic mechanical challenges to which they respond by remodeling their cytoskeleton. In cancer, these encounters are often altered thereby causing a change in the cellular response. Here we have been employing atomic force microscopy (AFM) which allows for measuring changes in the nanomechanical properties associated with tumorigenic transformation under near-physiological conditions. For example, the local stiffness of a specimen may be determined at high spatial resolution by indenting the specimen surface with the AFM tip and monitoring its deformation. On a nanoscopic level, AFM has been recently used in a clinical setting to probe with high sensitivity cell mechanics. To date, AFM has not yet been applied to three-dimensional (3D) cell culture models or intact cancer tissue, which more appropriately represents the In Vivo characteristics of the disease. Towards this goal, we have grown normal (wt) Rat2 fibroblasts and a tumorigenic derivative (Rat2sm9) in a 3D culture system to yield spheroids. Structural changes in the cytoskeleton of these cells cultured on a flat support or as 3D spheroids were correlated with differences in their nanomechanical properties. In tumor spheroids we measured a gradual centripetal softening from their periphery to the core caused by increasing hypoxia towards the core, whereas wt spheroids exhibited a homogeneous stiffness distribution. Nanomechanical testing of mammary tumors from transgenic mice corroborated these findings. To verify the clinical relevance of our study for human carcinomas, we obtained breast biopsies from 25 patients of different age groups and measured their nanomechanical properties. In malignant tumors the stiffness decreased up to 15-fold from the biopsy’s periphery to its core, whereas in benign lesions the stiffness appeared similar in all regions. The nanomechanical signature of the different carcinomas correlated well with the pathologist’s histological findings. Our long-term goal is to establish nanomechanical

775/B722
Activation of FAK by Integrin β4 in an EGFR/Src Dependent Manner in Tumor Progression.

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Both of integrin β4 and FAK (focal adhesion kinase) have been shown to associate with tumor progression in a variety of cancers. Recent studies showed that clustering of integrin β4 could lead to activation of FAK and its downstream signaling. Here, we further demonstrated a physical and functional association between these two molecules in Vivo and in vitro. An eleven amino-acid motif within the amino-terminus of FAK was identified to be essential for the interaction with the membrane proximal cytodomain of integrin β4. Nevertheless, this interaction occurred in an adhesion-dependent manner, especially adherent onto laminin, which subsequently resulted in the phosphorylation and activation of FAK in both of HCT116 and MDA-MB-231 cells. Moreover, EGF signaling and the activity of Src were crucial for this interaction, whereas PI3 kinase was indispensable. Interestingly, this interaction was not observed in MCF7 and other less-aggressive tumor cells, implicating that the involvement of this interaction in tumor progression. In addition, in HCT116 and MDA-MB-231 cells, disruption of the interaction between integrin β4 and FAK reduced cell proliferation, migration and invasion, in accordance with the roles of phospho-Akt, phospho-Stat3, and phospho-p38 resulted from this interference. Our data resolved for the first time a direct signaling transmission from integrin β4 to FAK through the physical recruitment in regulating cellular functions, thereby strengthening a link between integrin and FAK biochemically and functionally and shedding light on better strategies for cancer therapies. Key words: integrin beta4, FAK, tumorigenesis, metastasis

776/B723
Regulation of Hoxa1 Signaling during the Endodermal Differentiation of Mouse ES Cells.

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Embryonic stem (ES) cells have the ability to differentiate into a wide variety of somatic and extra-embryonic tissues. ES cells can differentiate into all three primary embryonic germ layers (ectoderm, mesoderm, and endoderm). Differentiations of ES cells into embryonic endodermal lineages are poorly understood. The endodermal differentiation of ES is directed by the expression of the master gene Sox17. Previous experiment in our lab indicates Hoxal gene to inhibit the endodermal differentiation in mouse ES cells by repression the expression of Sox17 gene but the exact mechanism is unknown. Thus, the goal of this research is to understand the mechanism by which the Hoxal transcription factor represses the differentiation of ES cells into endodermal precursors and to identify which retinoic acid receptor (RAR α, β, or γ) is involved in the activation of endodermal markers. Investigating the mechanism by which Hoxa1 represses the differentiation cell along the endodermal lineage, was accomplished by examining the role of Hoxa1 protein first as a direct repressor of endodermal gene expression and then as a direct activator of neural gene expression. To examine the role of Hoxal as a direct repressor of endodermal differentiation, the time-dependent expression of both Hoxa1 and Sox17 mRNA expression in Wt embryoid bodies (EBs) was determined by RT-PCR after treatment with various RA doses. To identify which of the RARs is involved in the activation of Sox17 gene expression by RA, we treated EBs produced from Wt and Hoxa1-/- Es cells with various concentrations of selective RAR agonists. The expression levels of Sox17 mRNA were assayed by RT-PCR. The results obtained from this work may help to improve current endodermal differentiation protocols employing ES cells and, therefore, it would impact a number of related research areas including tissue engineering.

777/B724
Improving Sialylation of Recombinant Glycoproteins in Cultured Mammalian Cells Using a Pathway-Focused Glycogene Toolbox.

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Sialylation extent of N-glycans critically determines the circulatory half-lives of recombinant glycoprotein drugs. Significant efforts have been made to improve the sialylation of recombinant glycoproteins with the aim of extending their in vivo circulation time, which primarily focus on the glycan terminal modifying enzymes. Here we report a systematic functional analysis of 31 major N-glycosylation-related genes, which form the “glycogene toolbox”, on sialylation of recombinant EPO expressed in six commonly used mammalian cell lines. The sialylation pattern of recombinant EPO was determined by isoelectric focusing/immunoblotting. Among these lines, BHK and CHO cells were found to sialylate recombinant EPO most effectively. HEK293, Cos-7 and 3T3 cells showed intermediate sialylation capabilities, whereas NS0 cells sialylated recombinant EPO poorly. Combinatorial and individual overexpression of the glycogenes showed that none of the 31 genes was able to improve EPO sialylation in BHK cells and CHO cells. In HEK293, Cos-7, 3T3 and NS0 cells, however, two sialyltransferases, namely ST3GalIII and ST3GalIV, significantly enhanced the extent of recombinant EPO sialylation. ST6GalI overexpression was also able to enhance recombinant EPO sialylation in HEK293 and Cos-7 cells, though to a lesser extent. In contrast, overexpression of the upstream glycogenes that synthesize the core structures of N-glycans had no positive impact on EPO sialylation in all six cell lines tested. These upstream genes did not have a synergistic effect when co-transfected with sialyltransferases. Our pathway-focused functional analysis of glycogenes provides systematic insights into impacts of glycogenes on sialylation of recombinant glycoproteins and a general guide for engineering mammalian cells to produce highly sialylated N-glycoproteins.

778/B725
Mutation in Leucine 118 of the LHβ Subunit Reveals a Novel Sorting Signal for the Regulated Secretion of LH from GH3 Cells.

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The pituitary lutropin (LH) and follitropin (FSH) are members of glycoprotein hormone family, which also include thyrotropin. The coordinated secretion and activities of LH and FSH are essential for control of gonadal function. LH dimer is released through the regulated pathway, i.e., it is released by secretagogues, while FSH dimer is secreted constitutively. Defining the sorting signals governing the trafficking of LH and FSH is critical for understanding the link between their secretion and reproductive role. Since LH and FSH are synthesized in the same gonadotrope cell and share an identical α subunit, the β subunit must contain the determinants for sorting. One unique feature of the LHβ subunit is its carboxyl terminal hydrophobic heptapeptide (115-Leu-Ser-Gly-Leu-Leu-Phe-Leu-121). Previously we demonstrated that deleting the heptapeptide from LHβ subunit diverted the truncated LH dimer to the constitutive pathway, resulting in a significant decrease in the accumulated intracellular pool in transfected rat somatotrope-derived GH3 cells. Addition of the heptapeptide to FSHβ resulted in a more regulated secretion of the FSH dimer proving this peptide is a sorting signal for LH dimer. The sequence resembles the XXXLeuLeu motif found in the cytoplasmic domain of transmembrane proteins. To examine the structure-function of the heptapeptide several alanine substitution mutants spanning residues 118-121 were constructed. Stable GH3 cell lines were labeled with [35S]cysteine and proteins from lysates and media were immunoprecipitated with antiserum that reacts with human LHβ. Absence of leu118 or both leu118 and 119 in the heptapeptide not only decreased the accumulated intracellular pool of the mutants, but also dramatically enhanced their secretion compared to the LH dimer. These results suggest that the leucine residues in the heptapeptide of the LHβ subunit, particularly residue 118, are critical determinants for the regulated secretion of LH dimer.

779/B726
Combination of 5-Azacytidine Plus Ascorbic Acid Induces Morphological Features and Molecular Markers Like-Cardiomyocytes in Mesenchymal Stem Cells from Bone Marrow.

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Cardiovascular diseases are the most frequent cause of death in the industrialized world, with the main contributor being myocardial infarction. Regeneration of the diseased myocardium by cell transplantation is an attractive therapeutic modality. Bone marrow mesenchymal stem cells have been shown to transdifferentiate into cardiomyocytes after 5-azacytidine treatment (5-aza). Nevertheless, an important goal is to develop efficient protocols for directing the cardiomyogenic differentiation In Vitro so many researches look for new suggestions to improve the conditions actually exist. The objective of this work was the isolation and characterization of mesenchymal stem cells from rat bone marrow as well as their differentiation in vitro. We show that exposure of the cells to a combination of two differentiate factors mainly mentioned in literature: 5-azacytidine and ascorbic acid, induce differentiation of cells producing morphology like cardiomyocytes (cells with defined fibers and a more extended shape). Methods. We employed microscopy and RT-PCR to confirm that treatment with 5-aza plus ascorbic acid induces a loss of the morphology and molecular markers of mesenchymal cells, such as CD70 and SH2 (depending of the concentration). This effect was not observed with the factors by separate. We show that the combination of both factors induce the gene expression of markers of cardiomyocytes such as cardiac troponin T (cTnT) as well as Nkx2.5. Conclusions: The capability of differentiation of mesenchymal stem cells from bone marrow into cardiomyocytes using a combination of 5-azacytidine-ascorbic could be approach for cardiac tissue repair and could represent a great promise for clinical application.

780/B727
Effect of Methyl Nutrients on the Growth of Multidrug Resistant MCF-7 Human Breast Cancer Cells.

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Lipotropes (methyl group containing nutrients, including methionine, choline, folate, and vitamin B12) are dietary methyl donors essential for epigenetic changes required for cell proliferation and maintenance of tissue integrity. The objective of this research was to determine the extent to which dietary methyl donors may affect the proliferation and death (caspase-3 enzyme activity) of the multidrug resistant MCF-7 (MCF-7/Adr) and the parent MCF-7 human breast cancer cells. MCF-7 and MCF-7/Adr were allowed to grow in a complete Dulbecco's Modified Eagle Medium in a tissue culture flask. Upon reaching confluence, cells were seeded in four 96-well flat-bottom tissue culture plates and treatment cells exposed to 2.5, 5, 7.5, 10, or 20 times the level of each lipotrope contained in the basal culture medium (control) and then incubated for 24, 48, 72, or 96 hours. Cell proliferation was measured using colorimetric CellTiter 96 Aqueous One Solution Assay. Cells were also seeded on a 24-well tissue plate, treated with or without the 20 times lipotrope level, and cell lysates collected after 120 hours. The activity of caspase-3 enzyme was measured by the colorimetric assay with CaspACE assay system. After 96 hours of incubation, lipotropes reduced the proliferation of MCF-7/Adr and MCF-7 by 17% and 5%, respectively, as compared to the control. Lipotropes also slightly increased the activity of caspase-3 in both cell lines as compared to the control. These results suggest that dietary methyl nutrients may alter the expression of genes conferring multidrug resistance in human breast cancer cells, thereby rendering the cells susceptible to apoptosis. Further studies on the effect of lipotropes on the expression of genes responsible for multidrug resistance (e.g., MDR1) and the synergistic effect of lipotropes with cancer drugs on resistant cells are warranted.

781/B728
A Quantification of Resting Membrane Potential Changes in E. coli and HEK293-Herg Cells by Dielectric Spectroscopy.
We present a method to obtain the resting membrane potential ($\Delta \Psi$) from the dielectric behavior of a suspension of living cells by the use of dielectric spectroscopy (DS). The presence of membrane potential has a specific effect on the dielectric behavior of cell suspensions and it can be extracted from the low frequency dielectric dispersion curves. Therefore, we use DS to monitor low frequency dielectric permittivity changes of E. coli and mammalian cell suspensions from HEK293-hERG line. E. coli cells are harvested from three regions of the exponential growth stage: early, mid and late phase. For all DS recordings, cells were measured at the same concentration, but were harvested in different growth stages and resuspended in water with glucose. From the dielectric curves, the average $\Delta \Psi$ of E. coli is extracted by means of a theoretical model. Membrane potential values resulting from fittings with the theoretical model indicate that membranes depolarize from -220 mV (early exponential phase) to -140 mV (late exponential phase). For mammalian cells, $\Delta \Psi$ changes are triggered by the use of various pharmaceutical compounds that act as HERG K+ channel blockers and IC50 values are computed for each compound.

782/B729
A Novel Marker of Undifferentiated Liver Progenitor Cells.
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Objective: Although some of hepatic progenitor cells have been characterized, up to now, neither liver stem cells nor universal liver stem cell markers have been identified. An adult rat liver stem cell line, Lig-8, has been derived from non-parenchymal fraction of F344 rats. This study is to identify and characterize interest rat stem cell marker, LigAg, which is specifically express in Lig-8 cells and recognized by our derived monoclonal antibody, named Ligab. Methods: to generate monoclonal antibody Ligab, we subcutaneously injected whole Lig-8 cells into mice. The Ligab specifically recognizes its target antigen (LigAg) with native form expressed by Lig-8 cells. for knowing the presence of LigAg in Lig-8 cells and the localization of LigAg-bearing cells in rat liver, we indirectly conjugated Ligab with FITC to perform immunofluorescent stain. for In Vitro differentiation, sodium butyrate (SB) was used to induce Lig-8 cells differentiation, and differentiated properties of expression of cytokeratin (CK)-19 and albumin were detected by PCR and immunocytostaining. To identify LigAg, we extracted different fractions of proteins from Lig-8 cells followed by immunoprecipitation and analyzed with SDS-PAGE. The identified bands in SDS-PAGE will be excised for peptide sequencing. Results: The yielded Ligab belongs to IgG subclass G1. LigAg is expressed on membrane and in cytoplasm of Lig-8 cells. Ligab could detect its antigen only by native PAGE-based immunoblotting. Indirect immunohistochemical staining in rat normal liver sections distinguishes LigAg bearing cells from currently known hepatic progenitor cells. In the induction with SB in vitro, Lig-8 cells were shown able to differentiate into both hepatocytes and bile duct cells with prospective markers expression. More interestingly, LigAg was decreasingly expressed along with Lig-8 cells differentiation. Conclusions: Inasmuch as our results showed LigAg was specifically expressed in hepatic progenitor cells, and its expression in a decreasing trend with Lig-8 cells differentiation. LigAg can be a differentiation-related marker for these cells and Ligab may help study the molecular mechanisms of liver progenitor cell differentiation.

783/B730
Silencing TIEG1 Expression Promotes Myotube Formation through Regulation of Apoptosis.
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TGF-β inducible early gene 1 (TIEG1), also called KLF10, is a zinc-finger transcription factor and regulates cell proliferation, differentiation and apoptosis in osteoblasts and leukocytes. It has been reported that TIEG1 expresses highly in skeletal muscle and the expression was induced by TGF-β, one of myogenic inhibitor. However, there are no any studies on the role for TIEG1 in skeletal muscle. Therefore, we attempted to define the expression and function of TIEG1 during myogenesis using C2C12 myoblasts. The expression of TIEG1 mRNA was significantly up-regulated in the early period and the increased expression maintained during myogenic differentiation. TGF-β and myostatin, an inhibitor against myogenesis, induced the TIEG1 mRNA expression at the early stage of proliferation. When TIEG1 expression was silenced using siRNA, TIEG1 mRNA substantially declined. Silencing TIEG1 expression did not affect the cell number and the cell proliferation by BrdU incorporation assay. on the other hand, TIEG1 silencing cells significantly augmented myotube formation (fusion index) and nuclear number per field compared with control cells. These findings indicate that myogenic differentiation may be promoted by TIEG1. However, no differences detected on the mRNA expressions of myogenin, myogenic differentiation marker, between TIEG1 silencing and control C2C12. In contrast, vital cell number in TIEG1 silencing cell cultures predominated over control cell cultures in the early stage of differentiation. Since myoblasts undergo apoptosis during myogenic differentiation, apoptotic cells were determined in TIEG1 silencing cell cultures using TUNEL staining. The proportion of apoptotic TIEG1 silencing cells was less than that of control cells. However, caspase-3 activity was expressed in TIEG1 silencing cells higher than control cells. The results indicate that TIEG1 regulates myoblast apoptosis during myogenic differentiation independently of caspase pathway, resulting in inhibiting myotube formation.

784/B731
Study of Cytotoxic Effect of Photodynamic Active Drugs on NIH3T3 Cell Line Morphology.
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In presence of molecular oxygen, light-activation of the photosensitizer, which accumulates in cancer cells, leads to the local production of reactive oxygen species (ROS). This is one of the several reasons leading to cell death and in some cases we could observe signs of apoptosis. We study changes in the morphology of non-cancer mouse fibroblast cell (NIH3T3) before-after photodynamic therapy (PDT) by atomic force microscopy (AFM), scanning electron microscopy (SEM), fluorescent microscopy and cytotoxic influence determined by measuring of reactive oxygen species production (ROS). We use the CAlIPcS2 an ZnTPPS4 as photodynamically active components. PDT was induced by efficient LED source (670 nm and 417 nm, 12 mW.cm-2) with light dose up to 15 J.cm-2. The kinetic production of ROS was study by molecular probe CM-H2DCFDA and fluororeader Biotech. Fluorescent microscopy was undergoing by Annexin/propidium measuring. Samples for AFM and SEM were prepared using glutaraldehyde fixation and scanned in air. The results show ROS kinetic production during PDT and modification of morphological features investigated by AFM. The combination of a sensitizer and the specific light source can lead to the loss of surface rigidity and eventually to dramatic changes of the cell shape, which we can study by different microscopic methods with high resolution. Our result indicate that non-cancer NIH3T3 cell line, are not so sensitive to PDT as cancer cells. This work was supported by the Ministry of Health NS9648-4/2008 and Ministry of Education of the Czech Republic MSM 6198959216.

785/B732
JNK and ERK Regulate TGF-β1 and FGF2-Induced Downregulation of Lumican and Keratocan Expression in Corneal Keratocytes.
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Corneal stroma cells (keratocytes) are quiescent in the normal adult cornea. However, they are activated to fibroblast or myofibroblast phenotypes in response to a stromal injury. Downregulation of the expression of keratan sulfate proteoglycans (KSPGs), lumican and keratocan, is one of the phenotypic changes associated with activation of keratocytes. TGF-β1 or FGF-2 induces downregulation of lumican and keratocan In Vitro in cultured corneal stromal keratocytes. The present study evaluated whether MAPKs regulate TGF-β1 or FGF2-induced downregulation of KSPGs. Keratocytes isolated from rabbit corneal stroma, plated in a serum free medium, were activated with FGF2/heparin or TGF-β1 in the presence or absence of inhibitors of MAPKs, JNK, ERK, or p38. Immunocytochemical analysis of the cells and western blot analysis of secreted proteoglycans indicated that the inhibition of JNK with SP600125 or ERK with PD98059 during keratocyte activation prevented TGF-β1 or FGF2-induced downregulation of KSPGs. Similar analyses of the core proteins derived by keratanase digestion of the KSPGs secreted in the culture supernatants indicated that both lumican and keratocan were downregulated in the activated keratocytes. JNK or ERK inhibition blocked downregulation of both lumican and keratocan. Real-time RT-PCR analysis of mRNAs indicated that the levels of lumican and keratocan mRNAs were significantly reduced in TGF-β1 and FGF2 activated keratocytes. JNK and ERK inhibition during the activation prevented the loss in the levels of lumican and keratocan mRNAs. In conclusion, activation of JNK and ERK signaling pathways may be responsible for downregulation of the transcription of lumican and keratocan mRNAs and thus the diminished expression of these KSPGs during corneal wound healing. (Supported by NIH grants EY03263(NS), EY08098 (core grant), Research to Prevent Blindness, and Eye and Ear Foundation of Pittsburgh, PA.)

786/B733
Study of Photodynamic Effect on MCF7 Cell Lines by Fluorescence Methods.
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Photodynamic therapy (PDT) utilizes a combination of a photosensitizing chemical and visible light for the treatment of solid malignancies and is also showing promise as a treatment modality for many nonmalignant diseases including atherosclerosis deposits inside arteries. The photochemical interactions of sensitizer, light, and molecular oxygen produce singlet oxygen and other forms of active oxygen, such as peroxide, hydroxyl radical and superoxid ion. The tumour is destroyed either by reactive singlet oxygen species (ROS), type II mechanism, and radical products, type I mechanism, generated in an energy transfer reaction. Phthalocyanine CIAIPcS2, belonging among the promising second generation of sensitizers, was evaluated as an inducer of photodamage and apoptosis on MCF7 (human breast adenocarcinoma) cell line. The light emitting diodes (LEDs 670 nm, FWHM 15 nm, 10 mW.cm-2) for identification of apoptosis the Vybrant apoptosis Assay Kit (annexin V conjugated to fluorescein and propidium iodide) was used. Morphological changes in cells have been evaluated using fluorescent microscope. The production of ROS was investigated by molecular probe CM-H2DCFDA. Cell damage was evaluated using fluorescence microscope. The quantitative ROS production changes in relation to sensitizer concentration were proved by fluororeader. Efficiency of photodynamic therapy is affected by a number of factors including absorption spectrum of the photosensitizer, wavelength of the activation light, depth of the light in the biological tissue, tissue answer on singlet oxygen. The ability of PDT to produce a rapid apoptot ic response may be an important element of successful photodynamic therapy. MCF7 cells are sensitive to photodynamic damage. Phthalocyanine CIAIPcS2 represents efficient sensitizer with high phototoxicity to MCF7 cells. This work was supported by the Ministry of Health NS9648-4/2008 and Ministry of Education of the Czech Republic MSM 6198959216.

787/B734
Effect of Co-Culture with Chondrocytes on Adipose Tissue-Derived Mesenchymal Stem Cells.
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INTRODUCTION Adipose tissue-derived mesenchymal stem cells (ATMSCs) obtained from lipoaspirates have the multi-lineage potential to differentiate into adipogenic, chondrogenic, myogenic, and osteogenic cells. However, several studies indicated that ATMSCs have lower chondrogenic potential compared with MSCs from bone marrow. The aim of this study was to test the hypothesis that co-culture of ATMSCs with chondrocyte or culturing ATMSCS under conditioned medium from chondrocytes might promote chondrogenesis from ATMSCs.

METHODS To examine cell-cell interactions in co-culture, in-vitro pellet cultures were carried out by mixing 1.25 x 10^5 ATMSCs (P3) and 1.25 x 10^5 chondrocytes (P3) in the same pellet, and compared with the pellets from 2.5 x 10^5 ATMSCs or from 2.5 x 10^5 chondrocytes. These were cultured in chondrogenic medium that did not contain growth factors. In order to effect of conditioned media from chondrocytes culture on ATMSCs, 2.5 x 10^5 ATMSCs were cultured in soups (soup: conditioned medium from chondrocytes culture). RESULTS The pellets were digested after 3 weeks of culture, and the DNA contents and GAG were measured. GAG contents increased from ATMSCS either by co-culturing with chondrocytes (10.7%, p<0.05) or by culturing under conditioned medium from chondrocytes culture (9.6%, p<0.05). Real-time PCR analysis showed that COL2A1 slightly increased by 20% when ATMSCs were treated with conditioned medium from chondrocytes culture. COL1A1 gene expression was stationary and COL10A1 gene expression slight increased in the conditioned medium. Although SOX-9 and COL2A1gene expression was several times greater in the ATMSCs-chondrocytes co-culture than in ATMSCs only, these gene expressions were still several times lower than those in chondrocytes only. Interestingly, COL1A1 and COL10A1 gene expressions were greater in ATMSCs-chondrocytes co-culture than in ATMSCs only. The results from Safranin-O staining generally paralleled those from GAG analyses: greater metachromatic staining when treated with conditioned media or in ATMSCs-chondrocytes co-culture.

Analyzing the Evolution of the Melanocortin Receptor Accessory Protein Gene Family.

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The human melanocortin 2 receptor (MC2R), unlike the other human melanocortin receptors (MC1R, MC3R, MC4R, MC5R), requires the melanocortin receptor accessory protein (MRAP) for proper routing from the ER to the plasma membrane. There also is evidence that MRAP plays a role in the signal transduction process when ACTH binds to mammalian MC2Rs. MRAP is single chain polypeptide with a single transmembrane spanning domain. In the human genome there are two mrap-related genes (mrap and mrap2) which are located on different chromosomes. Working on the assumption that all vertebrate MC2Rs require an “MRAP” for translocation and activation, genomes from several vertebrates were screened for the presence of MRAP-related genes using the tblastn program. The MRAP gene was detected in all of the mammalian genomes that were analyzed and in the genome of the chicken (Gallus gallus). However, this gene was not detected in genomes of a reptile, an amphibian, two bony fish or a cartilaginous fish. By contrast the MRAP2 gene was detected in all vertebrate genomes analyzed. Following alignment of the MRAP-related and MRAP2-related proteins, Maximum Parsimony analysis (PAUP) indicated that the MRAP-related and MRAP2-related proteins formed two distinct clades. Collectively, these data suggest that MRAP2 was the original melanocortin accessory protein gene, and that the divergence of the MRAP gene from the MRAP2 gene occurred during the radiation of the reptiles. To test the functional role of MRAPs for non-mammalian MC2Rs, the MC2R gene of the frog, Xenopus tropicalis, was transfected into CHO cells in the presence or absence of either mouse MRAP or mouse MRAP2. The frog MC2R only translocated to the plasma membrane when the mammalian MRAPs were co-transfected with the frog MC2R. It appears then that the evolution of a functionally distinct vertebrate MC2R gene has been dependent on the co-evolution of the MRAP genes.

Collagen Based 3-D Tissue Culture Models.
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Introduction Three-dimensional (3-D) tissue models are best suited for In Vitro studies of cellular responses that represent In Vivo relevance. Particularly important is their suitability for studying processes that In Vivo involve the preparation of tissue for mechanical challenges such as ossification and mechano-transduction. Purpose to demonstrate that impregnation with collagen type I gel containing mesenchymal stem cells ("coll-gel"), of porous scaffolds of differing stiffness serves as appropriate 3-D model for studies of bone maturation and characteristics. Methods Collagen type I foam and βTCP Preforms®, were impregnated with "coll gel" containing fluorescently labeled human dermal fibroblasts or human mesenchymal stem cells (hMSC). The cells were visualized using confocal microscopy. Treatment of the matrices with collagenase released the cells and allowed determination cell viability and proliferation using MTT. Response of cells to mechanical forces was also studied. Ossification of impregnated scaffolds by hMSC after osteogenic differentiation was demonstrated by von Kossa stain. Results Impregnation of collagen foam and βTCP showed 50% and 50% absorption of their weight in collagen solution (gel) respectively. Cy5 labeled collagen type I occupied the pores throughout the two scaffolds. Long-term viability (30 days), migration of dermal fibroblast into and out of the impregnated collagen foam, and response of cells to stretching were demonstrated. Matrices containing collagen gel populated with hMSC supported were von Kossa stain positive (ossification) and contained deposited glycosaminoglycans. Conclusions The scaffolds retain collagen type I as a fibrillar hydro-gel and contained randomly distributed cells throughout. Long-term cell survival allowed differentiation in situ. The model was cytocompatible as demonstrated by proliferation and migration of the cells into and out of the matrix. Alignment of the cells in response to applied forces suggested mechanoresponsiveness, and ossification indicated In Vivo relevance. Taken together these observations suggest that the proposed strategy to be appropriate for studying bone morphogenesis and mechano-responses in vitro.

790/B737
Non-Viral Transfer of Sox-Trio Gene to Adipose Stem Cells Using a Microporator.
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INTRODUCTION : It has been reported that adipose tissue-derived mesenchymal stem cells (ASCs) obtained from lipoaspirates have the multilineage potential to differentiate into adipogenic, chondrogenic, myogenic and osteogenic cells. However, several studies including one from the authors’ group demonstrated that chondrogenic differentiation comparable to that from MSCs was not achieved from ASCs. This study tested the hypothesis that Sox-trios(Sox-5,6,9) enhance chondrogenesis and suppress hypertrophic changes during chondrogenesis from ASCs (adipose tissue stem cells). METHODS : to create non-viral expressing Sox5, Sox6, and Sox9, full-length human Sox5, Sox6, and Sox9, full-length human Sox5, Sox6, and Sox9 complementary DNA (cDNA) was amplified by polymerase chain reaction (PCR) and cloned into pEGFPC1 mammalian expression vector. The microporatorTM and the buffer system were utilized for gene delivery. After microporation, in-vitro pellet cultures were carried out using 2.5 x 10⁵ ASCs in chondrogenic medium. Flow cytometry revealed that 85~98% of cells were infected with Sox-trio genes. After three weeks, cells were analyzed for DNA contents, GAG amount, real time PCR, western blotting and Safranin-O staining. RESULTS : GAG contents increased 3-folds when Sox-trio was transferred. Real-time PCR analysis showed that the mRNA levels of COL2A1 increased 8-folds in ASCs to which Sox-trio genes were transferred. COL1A1 or COL10A1 mRNA did not change, in contrast to ASCs in which TGF-β2 was treated. Safranin-O staining demonstrated that ASCs to which the sox-trio genes were transferred exhibited greater accumulation of proteoglycan-rich matrix than ASCs treated with TGF-β2. Western blotting showed that ASCs to which the sox-trio genes were transferred exhibited greater level of type II collagen than ASCs treated with TGF-β2. DISCUSSION : This system of nonviral Sox-trio gene transfer, with high efficiency for transfection and great potential for the inductions of chondrogenic differentiation from MSCs, offers a powerful means for cartilage tissue engineering from ASCs.
Polyphenolic Stilbenes Suppress TPA/VD3-Induced Osteoclastic Changes of Monocytic Cells through NF-κB Inhibition.

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Osteoclasts, resorbing bone and thus playing an essential role in bone remodeling and the pathology of osteoporosis, originate from monocyte-macrophage lineage, where NF-κB signaling is critical. As a polyphenolic stilbene resveratrol suppresses the NF-κB function, it is conceivable that it suppresses osteoclastic differentiation. In this study, we have examined effects of resveratrol and its analogs by using THP-1 monocytic cells as an osteoclastic model system. Exposure of the cells to 12-O-tetradecanoylphorbol-13-acetate (TPA) and 1α,25-dihydroxyvitamin D3 (VD3) for 3 - 5 days induced osteoclast-related phenotypes: attachment to the substrate and flattened, enlarged and/or multinucleated morphology. Phagocytic and tartrate-resistant acid phosphatase activities are also induced. for examination cells were treated with chemicals 4 - 5 h prior to TPA/VD3 and cultured in the presence of both reagents. Although resveratrol at 12.5 - 50 μM was not apparently effective, its analogs MR-4 (2.5 - 10 μM) and MC-4 (100 - 400 nM) [A.Gosslau et al., Eur J Pharmacol, 2008] substantially suppressed the osteoclastic changes: the flattened/enlarged cell population was reduced and there was a relative increase in small cells resembling untreated/induced cells. An NF-κB inhibitor BAY 11-7085 (5 - 10 μM) caused a similar change, suggesting that the effects of MR-4 and MC-4 are ascribable to NF-κB inhibition.

In addition, a Sirt1 inhibitor nicotinamide appears to be supportive for the osteoclastic changes. on the other hand, we have observed that heparin, a glycosaminoglycan species known to cause osteoporosis upon long-term clinical administration, augments the TPA/VD3-induced changes, where MEK/ERK and PI3K/AKT systems may be important. When cells were co-treated with heparin and MR-4 or MC-4, the stilbenes could not completely abolish the effect of heparin, and vice versa. The same results were obtained with a combination of heparin and BAY 11-7085. Therefore, the actions of heparin and the stilbenes may employ different cellular signaling. These observations together imply that the polyphenolic stilbenes MR-4 and MC-4 have ability to reduce osteoclasts through NF-κB inhibition.

Raman Spectroscopic Analysis of Rat Pancreatic Islets.

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Raman spectroscopy is an established technique that is now being innovatively used as a method for characterizing chemical properties of tissues and cells. Thus in these preliminary studies we explore the feasibility for the use of Raman spectroscopy in differentiation and functional studies of pancreatic islet cells. In addition, procedures involving pancreatic islet cells will be examined using the RDABS® Muscarinic M₃ Receptor IFA kit for indirect immunofluorescence. In these studies, alloxan monohydrate (C₄H₂N₂O₄ • H₂O) was used to induce the hyperglycemic condition in Sprague Dawley rats. Islets were isolated using a liberase enzyme solution prepared in Hank’s Balanced Salt Solution buffer which was injected into the common bile duct to distend the pancreas. The distended pancreas was digested with liberase enzyme and islet cells were purified using a Ficoll gradient. Islets were then handpicked using an inverted surgical microscope and plated in Dulbecco’s modified Eagle’s medium without sodium pyruvate. Cultures were supplemented with 10% heat-inactivated, fetal calf serum and 1% gentamicin and were incubated at 37°C in a 5% CO₂ environment. Islet specificity was determined by the use of dithizone (DTZ) stain which binds to the Zn²⁺ molecule of insulin revealing a red color. Photomicrographs were taken of freshly isolated, alloxan treated, and the DTZ stained islets using a Nikon Eclipse E600 Biomedical Microscope. Also, isolated islets were placed on an excimer laser optic chip, examined using a Horiba Jobin Yvon HR 800 Confocal Raman Spectrometer, and scanned using a He-Ne 635 nm laser. Preliminary results using Raman
spectroscopy revealed a significant peak analysis at a Raman shift of approximately 3000 cm\(^{-1}\). To our knowledge, Raman spectroscopy has never been used to examine rat pancreatic islets under these conditions and it is our goal to continue with these studies which may lead to the discovery of novel information about pancreatic function and a better understanding of conditions such as diabetes.

**793/B740**  
**A New Cellular Delivery System QD/Sr9: Exploration of the Efficiency, Uptake Mechanism, and Intracellular Localization.**  
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Recently luminescent semiconductor quantum dots (QDs) have begun to be used for delivering and monitoring biomolecules, such as drugs and proteins. The high quantum yield and photostability of QDs allows long term monitoring. However, we found that QDs alone had a very low efficiency to transport across cytoplasm membrane. In order to increase the delivery efficiency, we used nona-arginine (sR9), a cell penetrating peptides, to facilitate QDs' uptake. We found that sR9 could significantly increase the cellular uptake of QDs by non-covalent binding between QDs and sR9. Further, we explored the mechanisms of QD/sR9 internalization. Low temperature and metabolic inhibitors markedly influenced on the uptake of QD/sR9, indicating an energy-dependent process. Several pathway inhibitors and RNAi technique were used to analyze the transduction pathways. Epifluorescence imaging studies indicated that QD/sR9 were internalized by macropinocytosis, which was energy and lipid-raft dependent endocytosis process. According to results of RNAi, transduction was independent of clathrin- and caveolin-mediated endocytosis. Contrarily, data from inhibitors are not conclusive. Subsequently we explored the subcellular localization of QD/sR9, and found that the majority of QD/sR9 was localized in lysosomes, but not in mitochondria and nuclei. A pH sensitive, fusogenic HA2 sequence has been incorporated to liberate the delivery system from vesicle entrapment. These results demonstrated a new cellular delivery system. (Supported by M S&T cDNA Resource Center and NIH R15EB009530).

**794/B741**  
**Osteopontin Protein Expression and Secretion Patterns in HFOB 1.19 Cells Treated with Carbon Nanotubes and Chitosan-3-Trimethoxysilylpropyl Methacrylate.**  
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Biomaterials such as carbon nanotubes (CNT) and chitosan exhibit the characteristics necessary for successful bone regeneration. Osteopontin (OPN), a protein secreted by osteoblast cells, is a good indicator of normal bone cell growth. Therefore, our objective was to determine the expression and secretion patterns of OPN when human fetal osteoblastic cells (hFOB 1.19) were cultured with hybrid composite bone materials made from CNT and Chitosan-3-trimethoxysilylpropyl methacrylate (CTS-3-TMSPM). We believed that the aforementioned hybrid composite bone materials would increase the secretion and expression of OPN in the osteoblast cell line, hFOB 1.19. These osteoblast cells were treated with CNT or CTS-3-TMSPM (0, 250, 500 and 1000ng/mL) for 24, 48, and 72 hours. Media was collected daily and subjected to an enzyme linked immunosorbent assay (ELISA) to determine OPN secretion. Following 72 hours of treatment, cells were harvested and protein isolated for use in western blot analysis. Cells were also cultured on two chamber slides in the presence of CNT or CTS-3-TMSPM (0, 250, 500 and 1000ng/mL) for 24 hours and then subjected to immunocytochemistry (ICC) to localize OPN. The secretion of OPN by hFOB 1.19 cells was not significantly affected by CNTs or CTS-3-TMSPM. The expression of OPN protein tended to increase in a dose-dependent manner in cells that were grown in the presence of CTS-3-TMSPM. Alternatively, OPN protein expression tended to
decrease in a dose-dependent manner in CNT-treated cells. Osteopontin protein was localized in all cells, but there was a higher expression in cells that were grown in the presence of 500ng/mL of CNT compared to controls and CTS-3-TMSPM. Bone regeneration and repair may thus be aided by the use of CNT and CTS-3-TMSPM. Future work will be performed to determine the application of these biomaterials, CNT and CTS-3-TMSPM, for bone repair and regeneration.
Minisymposium 9: Cellular Basis of Morphogenesis (795 – 800)

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A Molecular Clutch Linking Actomyosin Contractility to Cell Movements.
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How embryonic cells transition from spatial patterning to morphogenesis is a fascinating and incompletely understood topic. In C. elegans, the first morphogenetic movement is the internalization of two endodermal precursor cells (E cells). The current model for how these cells become internalized is that an apically-enriched population of activated non-muscle Myosin II motors drives apical constriction, and this may pull a ring of six neighboring cells together to cover the free surfaces of the E cells. We have examined non-muscle Myosin II dynamics with diffraction-limited fluorescence imaging to follow movements of myosin foci with respect to the zones where E cells contact their neighboring cells. We expected to observe narrowing of the contact zones in concert with contraction of the actomyosin network. We were surprised to find instead that centripetal myosin movements preceded narrowing of contact zones, contracting the apical actomyosin network multiple times over before significant neighboring cell movements. Later, myosin foci continued to coalesce centripetally and contact zones narrowed in concert. This suggests that a regulatable link (a clutch) may connect cortical actomyosin contraction to neighboring cell movements. To test this hypothesis, first, we tracked cell surface movements using fluorescent quantum dots. Our results suggest that free surfaces of E cells move together with cortical actomyosin contraction before neighboring cells move in concert, suggesting that the regulatable link lies between the E cell apical cytoskeleton and neighboring cells, and hence may be comprised of cell-cell adhesion complex proteins or proteins that link the adhesion complex to the cytoskeleton. Second, we analyzed adhesion-defective embryos and found coupling of myosin and contact zone dynamics fails. Together with the finding that similar centripetal myosin movements move polarity proteins toward the center of the apical surface at earlier embryonic stages (Munro et al., 2004), our results suggest that the transition from apicobasal cell polarization to cell internalization is governed by a molecular clutch.

796
Sensory Dendrites Extend by ‘Dropping Anchor’ Prior to Cell Migration.
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One of the greatest unsolved problems in biology is how groups of cells self-assemble into highly ordered structures. Nowhere is this problem more astounding than in the nervous system, where neurons and glia assume exquisitely precise shapes that must be intimately coordinated for the nervous system to function. Amazingly, the C. elegans nervous system develops almost identically in each individual, implying that a map of every cell contact is encoded in the single-cell embryo. To decipher this map, we turned to the amphid, the major sense organ of C. elegans, composed of 12 neurons that extend unbranched sensory dendrites to the tip of the nose, and are ensheathed there by two glia. To learn how these cells attain their shapes, we isolated mutants in which sensory dendrites fail to reach the nose tip. These mutants defined two genes, dex-1 and dyf-7. Using single-cell tracking with a photoconvertible fluorescent protein, we found that sensory neurons born near the nose anchor dendritic tips there and then crawl away, stretching dendrites out behind them as they migrate; in dyf-7 mutants, the dendrites fail to anchor and are dragged along with the migrating cells. DEX-1 and DYF-7 are secreted and act during anchoring, with DYF-7 expressed by the neurons and localizing to dendrite tips, and DEX-1 expressed by neighboring cells. DYF-7 contains a zona pellucida (ZP) domain and forms multimers of dimers, likely assembling a matrix to which dendrites anchor. DEX-1 and DYF-7 resemble tectorins, matrix-forming proteins of the vertebrate inner ear that anchor the tips of sensory hair cells. We recently isolated a mutant in FBN-1, a fibrillin homolog with a ZP domain, which is also required for amphid dendrite anchoring, DEX-1, DYF-7 and FBN-1 are strongly required for the amphid but not other sense organs; likewise, we have found mutants that affect other sense organs but not
the amphid, suggesting each sense organ recognizes a discrete anchor site at the nose. As ZPs are found in diverse sense organs across species, and as the *C. elegans* genome encodes 40 ZP proteins, an intriguing hypothesis is that combinations of multimerized ZPs in the early embryo encode discrete anchor sites for different sensory dendrites.

797

*Drosophila* Cytonemes Are Ligand-Specific Cell Extensions That Ferry Signaling Molecules Over Long Distance.

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Cytonemes are types of filopodia first identified in the Drosophila wing imaginal disc that were proposed to be involved in long distance signaling during development. The present work reports presence of several novel types of cytonemes and shows that the sizes and orientations of each cytoneme type depend on a specific ligand protein. Receptors for ligands had punctal distributions that were motile and localized specifically in responding cytonemes and they appear to be localize only to responsive cytonemes. Vesicular puncta containing both co-localized ligand and receptor were found in cytonemes that appeared to touch ligand-expressing cells, providing direct evidence that cytonemes transporting signaling ligands. Thus, cytonemes are diverse in composition and function, can ferry signaling proteins, and exhibit specificity for stimulating ligands and as well as dynamic plasticity. These findings strongly support cytoneme-based movement of signaling proteins as a mechanism for cell-cell communication in which controlled amounts of signaling protein can be targeted to a specific recipient cell.

798

Planar Cell Polarity, Ciliogenesis, and Neural Tube Closure.

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The planar cell polarity (PCP) pathway controls a wide variety of polarized cellular processes during development. In vertebrate embryos, PCP proteins are essential for neural tube closure, acting to control both cell movements and ciliogenesis. Despite these important roles, the downstream mechanisms by which the PCP cascade directs the execution of discrete cell behaviors remains poorly understood. We have focused recent efforts on the PCP effector proteins, Fuz and Fritz. We show that Fuz acts at the level of vesicle trafficking during ciliogenesis, directing the transport of proteins to the basal body and along the axoneme. By contrast, Fritz interacts with cytosolic chaperones and controls microtubule assembly during ciliogenesis. Interesting, Fritz also acts to control plasma membrane stability during convergent extension. Understanding how Fuz and Fritz interact with the core PCP proteins such as Dishevelled is an active area of study in our lab. These data should shed light on the etiology of human neural tube defects. More generally, these results provide new insights into the mechanisms by which developmental regulatory systems interface with fundamental cellular machinery to drive morphogenesis.

799

Regulated Cell Polarity Defines Long Bone Morphology.

A. T. Dudley, M. J. Ahrens, Y. Li, H. Jiang; BMBCB, Northwestern University, Evanston, IL

A central question in developmental biology is how morphologically distinct structures are generated from similar cell types. The skeleton provides a good model system to answer this question due to the highly diverse and complex morphological differences displayed by the individual elements. Analysis of the growth plate cartilage by Dodds suggested that longitudinal growth of long bones might result from the specific arrangement of chondrocytes. In the proliferative zone, chondrocytes become discoid and arrange in columns, like stacks of coins, which are parallel to the long axis of the cartilage. Although well-organized chondrocytes derive
from a relatively unstructured pool of progenitor cells, it is not known whether this arrangement of cells is the result of a regulated process or is in response to physical constraints of the cartilage matrix. Here we test Dodds’ model that individual chondrocyte columns form from single progenitor cells by a process in which cells divide orthogonal to the stack then intercalate into columns. Here we show that discoid proliferative chondrocytes orient the division plane to generate daughter cells that are initially aligned orthogonal to the column and then intercalate. Downregulation of Frizzled (Fzd) signaling in chick long bones produces cell autonomous changes in proliferative chondrocyte organization characterized by arbitrary division planes and altered cell stacking. By contrast, in Piga mutant mice, chondrocytes display normal planes of cell division but fail to intercalate into columns. Thus, orientation of cell division and cell intercalation are mechanistically separable events. In all cases, defects in column formation lead to shorter wider bones, consistent with a model in which cell arrangement defines vectors of growth in developing cartilage. Collectively, our findings demonstrate that signaling pathways regulate cell polarity and cell arrangement in the cartilage growth plate and suggest important roles for polarized cell behaviors in generating the unique morphological characteristics that shape individual cartilage elements that compose the articulated skeleton.

800
Regulation of Oriented Cell Division by Receptor Tyrosine Kinase Signaling in Airway Morphogenesis.
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We have been studying the consequences of altering Receptor Tyrosine Kinase (RTK) signaling in the developing mouse lung by inactivating Sprouty genes, which encode negative feedback inhibitors of FGF receptor and other RTK signaling pathways. Our data show that the combined loss of sprouty1 and sprouty2 in lung epithelium at an early stage of development results in an increase in RAS/MAP Kinase (MAPK) signaling downstream of FGF10 and its cognate receptor, which causes widening and shortening of the primary airway branches. We present evidence that such alterations in airway size and shape are due to a change in oriented cell division within the lung epithelium resulting from the increase in RAS/MAPK signaling. These data thus support the idea that regulating airway morphogenesis depends on precise control of oriented cell division.

Minisymposium 10: Cilia and Centrosomes (801 – 806)

801
Evolution of Centriole Assembly Mechanisms.
Z. Carvalho-Santos1, F. Cadete2, P. Machado1, P. Branco1, A. Rodrigues-Martins1, J. Borrego-Pinto1, N. Matias1, J. Pereira-Leal2, M. Bettencourt-Dias1; 1Cell Cycle Regulation Lab, Instituto Gulbenkian de Ciência, Oeiras, Portugal, 2Computational Genomics Lab, Instituto Gulbenkian de Ciência, Oeiras, Portugal

The centriole Basal body (CBB) nucleates cilia and flagella and is an essential component of the centrosome, underlying eukaryotic microtubule-based motility, cell division and polarity. In recent years, components of the CBB assembly machinery have been identified, but little is known about the regulation and conservation of this important process. Given the diversity in cellular contexts encountered in eukaryotes, but remarkable conservation of CBB morphology, we asked whether we could obtain general mechanistic principles that explain CBB assembly. We profiled each component of the human CBB assembly machinery across eukaryotes. We found a universal module of three components (UNIMOD: SAS6, SAS4/CPAP and BLD10/CEP135) that correlates with the occurrence of CBBs and propose they define the CBB conserved morphological structure. Surprisingly, other players (SAK/PLK4, SPD2/CEP192 and CP110) emerged in a taxon-specific manner. Moreover, we often observed extreme protein divergence amongst CBB components and show that divergence amongst SAK/PLK4 family members leads
to loss of cross-species complementation, suggesting species-specific adaptations in CBB assembly. Our work defines universal predictors for the presence of CBBS, which we used to follow their evolution. We propose that the UNIMOD explains the conservation of CBB architecture and that taxon and tissue-specific molecular innovations play a role in coordinating CBBO biogenesis and function to different cellular contexts. In order to further understand the evolution of CBBS we have created a bioinformatics tool that allows integrating morphological information (annotated light and electron microscopy images) and molecular information (phylogenetic distributions of CBB components). By bridging the gap between morphological and molecular information and becoming a community built tool, CentrioleDB will enable a more general understanding of CBB assembly mechanisms.

802

**Poc1 Is Required for Basal Body Stabilization.**

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Basal bodies are responsible for the nucleation and anchoring of both motile and immotile cilia. Defects in basal bodies and cilia result in a class of human diseases known as ciliopathies. The biogenesis of basal bodies is facilitated by the step-wise addition of ultra-structural domains. Cartwheels, composed of nine-radiating spokes extending from a central hub, are formed early during basal body assembly. Triplet microtubules are then nucleated from the ends of each spoke to build the cylinder shaped structure. Using a proteomics strategy, we identified novel components of basal bodies, including the cartwheel protein, Poc1. Poc1 is a conserved WD40 repeat containing protein that is required for the structural maintenance of basal bodies in Tetrahymena. The two human Poc1-like proteins localize to the proximal end of centrioles and basal bodies and HsPoc1B knockdown decreases primary cilia formation. Poc1B knockdown in Zebrafish results in defects commonly associated with ciliopathies. To understand how Poc1 assembles into the basal body structure for stability, the incorporation of Poc1 protein during new basal body assembly was quantified. Proteins were found to localize at basal bodies by distinct mechanisms ranging from α-tubulin that is stably incorporated only during new assembly to those, such as Spag6, that dynamically exchange at basal bodies throughout the cell cycle. These patterns of incorporation reflect the diversity of ultra-structural domains that each component localizes. Poc1, among other core components that are responsible for basal body assembly and maintenance, stably incorporates during new basal body assembly. Finally, the timing of Poc1 incorporation relative to other basal body proteins was measured. These studies illuminate the molecular pathways for basal body assembly in the context of the basal body ultra-structure.

803

**Novel Features in Formation and Function of the “9+2” Axoneme Revealed by a Basal Body-Deficient Mutant of Chlamydomonas.**

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Axonemes of eukaryotic cilia and flagella have the “9+2” structure consisting of the nine outer doublet microtubules and the central pair microtubules (CP), with various projections periodically arranged on the microtubules. How this elaborate structure assembles and functions is poorly understood. To address this question, we investigated the effects of the change in the number of the outer doublets by observing axonemes produced by a Chlamydomonas mutant, bld12. This mutant has defects in stabilizing the 9-fold symmetry of the basal body structure, and thus produces axonemes with 8, 9, 10, or 11 doublets. Most of the 8-doublet axonemes contained no CP, but those of a double mutant with pf14, a mutant lacking the radial spokes, contained CPs. Thus the CP formation apparently depends on the space limited by the outer doublets and the radial spokes. In 10- or 11-doublet axonemes, 4 to 6 radial spokes were detached from the CP, resulting in a distorted arrangement of the doublets. The detachments were found predominantly
on the C2 side of the two CP microtubules, named C1 and C2. However, in double mutants with
pf6 or pf16, which lack a prominent projection on C1 or the entire C1 microtubule, radial spokes
frequently attached to a projection on C2. From these observations, we conclude that 1) the radial
spokes actually bind to the CP and pull the doublets toward the center, and 2) the projections on
C1 bind to the radial spoke more strongly than those on C2, while multiple projections on CP
interact with the radial spoke. These results thus provide invaluable insights into how the central
pair microtubules are formed and how they interact with radial spokes.

804
Cell Shape and Stiffness Regulate Ciliogenesis in Cell Cycle Arrested Cells.
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Background Regulation of the balance between cell growth and quiescence is fundamental to
embryogenesis and tissue renewal. Induction of cell quiescence is regulated by numerous factors
and coincides with dramatic cell reorganisation, including the growth of a primary cilium. The
relationship between cell cycle exit and primary cilium growth is poorly understood, though it is
believed that the two processes are tightly coupled. Results We analysed cell cycle exit and
ciliogenesis in human epithelial cells and found that all cells exiting the cycle do not generate a
primary cilium. Low incidences of primary cilia were observed in sparse cell cultures after cell
cycle arrest compared to confluent cultures. Using adhesive micro-patterns of various sizes, we
showed that the probability of generating a cilium was inversely correlated to the cell spreading
area. The growth of a primary cilium in sparse cell culture or in over-spread cells on micro-
patterns could be promoted by inhibition of Rho kinase suggesting that high level of acto-myosin
contraction prevents ciliogenesis. Consistent with this interpretation, most cells in sparse cultures
on soft, deformable surfaces had primary clia contrary to cultures on rigid substrates where few
cells were ciliated. In addition, cilia were longer in culture conditions reducing the level of cell
tension. Conclusions These results indicate that cell cycle exit is not sufficient to induce
ciliogenesis. External physical parameters affecting myosin phosphorylation and the level of cell
tension such as microenvironement geometry and rigidity regulate the growth and extension of
primary cilia in cell cycle arrested cells.

805
The BBSome Functions as a Coat Complex for Ciliary Membrane Trafficking.
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The primary cilium, a “signaling antenna” projecting at the surface of the cell, is required for the
transduction of Hedgehog and planar cell polarity signals and concentrates many signaling
receptors on its surface. Moreover, ciliary dysfunction leads to obesity, retinal degeneration and
kidney cysts in the inherited disorder Bardet-Biedl Syndrome (BBS). Although fourteen BBS
genes have now been identified, the molecular pathway of BBS has remained elusive. Our recent
discovery of a stable complex of seven highly conserved BBS proteins, the BBSome,
considerably simplifies the apparent molecular complexity of BBS. At the functional level, we have
implicated the BBSome in vesicular trafficking to the cilium based on its functional interaction with
Rab8, a trafficking GTPase required for cilium growth. However, a clear understanding of the
molecular activity of the BBSome is currently lacking. We have now initiated an in-depth
biochemical characterization of the BBSome and of the only other BBS protein that is universally
conserved in ciliated organisms, the Arf-like GTPase ARL6 BBS3. Using affinity
chromatography, structural modeling, gene replacement, and biochemical reconstitution from
pure components, we arrive at the conclusion that the BBSome functions as a vesicular sorting
complex that follows the paradigms established by clathrin, COPI and COPII coats.
Bardet-Biedl Syndrome (BBS) is a cilia-related disorder characterized predominantly by obesity and retinopathy. In humans, 12 genes causing BBS have been identified; 8 of these are conserved in Chlamydomonas. To learn more about the function of these genes, we identified Chlamydomonas insertional mutants defective in BBS1, BBS4, and BBS7. The mutants assemble motile, full-length flagella but lack the ability to phototax. In wild-type flagella, BBS4 is present in a ~12S complex (the BBSome) containing at least four other BBS proteins (BBS1, 5, 7, and 8) and undergoes intraflagellar transport (IFT). However, both immunofluorescence microscopy and double total internal reflection fluorescence microscopy using BBS4-GFP plus IFT20-mCherry (in a BBS4- and IFT20-null background) indicate that the BBSome is associated with only a subset of IFT particles. Absolute quantitation of IFT-particle proteins and BBSome proteins by mass spectrometry using an AQUA peptide strategy shows that the BBSome proteins are at least 5x less abundant than IFT-particle proteins in the wild-type flagellar membrane plus matrix fraction. In the bbs4 mutant, assembly and transport of IFT particles is unaffected and IFT complex a and complex B co-localize normally. 1D- and 2D-PAGE indicate that the mutant flagella contain normal amounts of most proteins but abnormally accumulate several signaling proteins, including a phospholipase. Consistent with this, the lipid composition of the membrane is altered in bbs4 flagella. We conclude that an evolutionarily conserved BBSome is carried by IFT but is not an integral component of the IFT machinery. Chlamydomonas BBS4 may be required for flagellar signaling and/or the removal of certain proteins from the flagellum via IFT.

Minisymposium 11: Host-Pathogen Interactions (807 – 812)

807

Molecular Mechanisms Underlying Pathogen Recognition in Plants.

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Plant disease resistance proteins belonging to the nucleotide binding-leucine rich repeat (NB-LRR) class often detect pathogen effector proteins indirectly, via sensing modification of effector targets. We are studying this process using the RPS5 protein from Arabidopsis. RPS5 detects the cysteine protease AvrPphB of Pseudomonas syringae, and this detection requires a second Arabidopsis protein, PBS1. Cleavage of PBS1 by AvrPphB is required to activate RPS5. Current work is focused on defining what parts of PBS1 are required for interaction with RPS5, what parts of RPS5 are required for downstream signaling, what other proteins are part of the RPS5 complex prior to activation, and whether subcellular relocalization of RPS5 occurs upon activation. Preliminary data indicate that RPS5 and PBS1 initially localize to the plasma membrane, but upon activation RPS5 relocates to the nucleus and to the chloroplast. We are currently evaluating the functional relevance of this relocalization. Our work has broad implications regarding activation of NB-LRR proteins in general, including human homologues belonging to the NOD family, which function in the human innate immune system, and which are implicated in several autoimmune diseases.
808
Exosomes as Regulators of the Immune Response against Mycobacterial Infections.
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*Mycobacterium tuberculosis*, the causative agent of tuberculosis, is a major human pathogen and is responsible for approximately 1.7 million deaths annually. It is estimated that over 2 billion people are infected with *M. tuberculosis* but the vast majority maintain a latent infection. Controlling an *M. tuberculosis* infection requires a robust innate and acquired immune response. However, *M. tuberculosis* is an intra-macrophage pathogen and it is unclear how antigens and pathogen associated molecular patterns (PAMPs) expressed by the bacteria gain access to the immune system to promote this robust response. We have found that macrophages infected *in vitro* and *in vivo* release exosomes containing mycobacterial components including lipoarabinomannan and the 19 kDa lipoprotein. Exosomes released from infected cells stimulate macrophage and dendritic cell activation in a TLR/MyD88 dependent manner. Moreover, exosomes isolated from *M. bovis* BCG infected macrophages when administered intra-nasally into uninfected mice stimulated antigen specific CD4+ and CD8+ T cells indicating that they have mycobacterial antigens. This T cell activation did not require co-administration of adjuvant suggesting that exosomes have both antigenic and adjuvant properties. Proteomic analysis showed that these exosomes contain over 50 mycobacterial proteins including a number of immuno-dominant antigens such as Ag85 complex, MPT-64, MPT-63 and GroES. Studies are ongoing to define the mechanism by which exosomes may function to regulate an immune response against a *M. tuberculosis* infection and whether exosomes can be developed into a TB vaccine and used in TB diagnostics.

809
A. E. Palmer, S. B. VanEngelenburg; Chemistry & Biochemistry, University of Colorado, Boulder, CO

The Type-III Secretion System (T3SS) is a sophisticated macromolecular machine enabling gram-negative pathogenic bacteria to inject proteins into eukaryotic host cells. Upon protein entry, T3SS effectors work cooperatively to hijack cellular signaling pathways and reprogram the host cell to enable bacterial survival. Progress in understanding when effectors are injected and where they localize within the host cell has been hindered by the lack of adequate tools to directly study these proteins in the native cellular environment. Moreover, studying the coordinated action of effectors has been hampered by the dearth of appropriate tools to track specific effectors within the context of infection. We have developed two approaches for lighting up and imaging bacterial effector proteins during the infection cycle, allowing us to dissect their secretion kinetics, localization, and dynamics within the host cell. We have applied these tools to Salmonella enterica Typhimurium. Salmonella enterica species encode two T3SSs and translocate over 60 individual proteins into host cells. Using an in situ chemical labeling system, we were able to quantify the rates of effector secretion into host cells using live-cell fluorescence microscopy, and demonstrated differential secretion for antagonistic effectors. In a complementary approach, to address the “where” of bacterial protein localization post-injection, we adapted a split GFP system which allows for live-cell monitoring of effector protein dynamics. Using these approaches we have defined the localization and dynamics of multiple Salmonella effectors during the course of an infection cycle. In particular we identified effectors that localize to distinct populations of Salmonella-induced filaments, which derive from different host-cell membranes.

810
Evolutionary Arms Races Involving the Innate Immunity Factor Protein Kinase R.
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Protein surfaces involved in host-pathogen interactions can evolve in a manner resembling molecular arms races where each side gains fleeting advantages in ongoing genetic conflicts. Signatures of these arms races can be discerned phylogenetically by calculating rates of non-synonymous codon substitutions (dN) - that alter amino acid sequences - compared to rates of synonymous, silent changes (dS). A dN to dS ratio exceeding one indicates positive selection, which can reflect rapid evolution in these antagonistic settings. We found that Protein kinase R (PKR), an important component of innate immunity in primates and other mammals, evolves under extreme positive selection in primates. This rapid evolution likely reflects conflicts with a diversity of viral factors that directly interact with PKR to disable it. For example, some viruses encode factors that alter the cellular distribution of PKR from cytoplasm to nucleus. Redistribution of PKR may be an effective means of blocking the activation of this antiviral kinase, which is triggered by the recognition of cytoplasmic nucleic acids produced by viruses. We examined PKR evolution at single codon resolution and discovered strong positive selection in an otherwise conserved helix, which makes direct contact with the substrate of PKR. Several viruses encode mimics of PKR’s substrate, so we tested the possibility that variation in PKR might affect vulnerability to such mimics. Performing infection studies in primate cell lines and using a yeast growth assay that recapitulates PKR activity, we tested a panel of primate variants of PKR against K3L, a substrate mimic from vaccinia virus. Some variants of PKR resist K3L due to positive selection in sites involved in substrate recognition. Surprisingly, variation in another surface of PKR also affects discrimination of substrate mimics, revealing that adaptive changes on multiple surfaces produces combinations of changes that increase the odds of defeating mimicry. Our study reveals exceptional evolutionary flexibility at a host-pathogen interface and provides a framework for examining other nucleic acid sensors and additional host factors that might evolve in molecular arms races with pathogens.

811
The Influenza Virus M2 Ion Channel Protein Mediates the ESCRT-Independent Budding of Filamentous Virions.
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Influenza a virus buds from cells as spherical (~100 nm diameter) and filamentous (~100 nm x 2-20 μm) virions that are thought to be equally infectious. Filamentous virions appear to be the predominant form isolated from the human upper respiratory tract and are seen in the recent 2009 H1N1 pandemic virus. While many of the interactions required for influenza virus assembly have been determined, the molecular machinery needed to complete the budding process for filamentous, as well as for spherical virions is not known. Many viruses utilize the host endosomal sorting complex required for transport (ESCRT) machinery to mediate membrane scission and virion release. Binding of late domain sequences in many viral matrix proteins mediates recruitment into the ESCRT pathway, enabling the virus to utilize host proteins involved in the budding and release of endosomal vesicles into multi-vesicular bodies, for their own budding. However, the available data for influenza virus indicates that the virus may bud independently of ESCRT proteins, with the mechanism of virion release unknown. While investigating the mechanism of filamentous virion budding, we have found a new role for the influenza virus M2 protein that is independent from its previously determined ion channel activity. Utilizing recombinant viruses and fluorescence microscopy, we show that multiple domains within the M2 protein are required for the formation of filamentous virions. We observed that the cytoplasmic tail of M2 is involved in localization of M2 to sites of virus budding. Through a variety of molecular and biochemical approaches, we find that the M2 protein’s amphipathic helix, located within the cytoplasmic tail, mediates a cholesterol-dependent alteration in membrane curvature. This alteration of membrane curvature is seen to facilitate filamentous virion formation and viral
budding. We suggest that M2 may mediate the final step of budding for all influenza a viruses, serving as a virus-encoded ESCRT substitute.

812
A. K. Neumann, K. Jacobson; Cell & Developmental Biology, University of North Carolina, Chapel Hill, NC

Fungal pathologies are seen in immunocompromised and healthy humans. C-type lectins expressed on immature dendritic cells (DC) recognize fungi. We report a novel dorsal pseudopodial protrusion, the “fungipod”, formed by DC after contact with yeast cell walls. These structures have a convoluted cell-proximal end and a smooth distal end. They persist for hours, exhibit noticeable growth and total 13.7±5.6 μm long and 1.8±0.67 μm wide at the contact. Fungipods contain clathrin and an actin core surrounded by a sheath of cortactin. The actin cytoskeleton, but not microtubules, is required for fungipod integrity and growth. An apparent rearward flow (225±55 nm/sec) exists from the zymosan contact site into the distal fungipod. The phagocytic receptor Dectin-1 is not required for fungipod formation, but CD206 (Mannose Receptor) is the generative receptor for these protrusions. These novel pseudopodial structures may be involved in yeast retention on the DC, promoting phagocytosis and engulfment responses to larger microorganisms.

Minisymposium 12: Lipid Dynamics (813 – 818)

813
Dynamics of Plasma Membrane Reorganization Induced by Equinatoxin II.
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Equinatoxin II is a pore forming toxin from sea anemone Equinia actinia that efficiently lyses several cell types and shows permeabilizing activity in model membranes. Equinatoxin II activity depends on the presence of sphingomyelin in the target membrane, which has been proposed to act as a specific receptor for the toxin and as a promoter of the membrane organization necessary for toxin activity. In this work, we investigated the mechanism of action of the toxin and the role of sphingomyelin in living cells. We found that fluorescently-labeled Equinatoxin II shows a higher binding to the apical membrane of polarized MDCK cells, which is enriched in sphingomyelin when compared to the basolateral membrane. Using 5D microscopy on living COS-7 cells, we followed the reorganization of the plasma membrane that we found to happen shortly after toxin binding. This redistribution affects raft and non-raft proteins labeled with GFP and leads to the formation of immobile protein domains. Interestingly, Equinatoxin II seems to colocalize with clustered GPI-GFP, a typical raft marker. Our fluorescence cross-correlation spectroscopy and FRET studies indicate that membrane reorganization is accompanied by Equinatoxin II oligomerization. at longer times, Equinatoxin II causes to the growth of non-retracting, plasma membrane blebs that correlate with calcium entry into the cytosol and PIP(4,5)P2 degradation at the plasma membrane. Finally, cells collapse after osmotic swelling. Altogether, our results suggest that alteration of the plasma membrane organization is part of the deadly strategy of Equinatoxin II and highlight the essential role of membrane structure for life progression.
Cell fusion is essential for organ development and sexual reproduction. Molecular genetic studies of cell fusion in *C. elegans* have led to the discovery of the FF family of fusogens. EFF-1 and AFF-1, are type I membrane glycoproteins that are essential for cell fusion. FF proteins can fuse cells when ectopically expressed on the plasma membranes of nematode, insect and mammalian cells. One third of all somatic cells in *C. elegans* fuse homotypically and specifically either via EFF-1 or AFF-1. Cells can change shape by fusing with themselves through a process of autacellular fusion. We will discuss how the FF proteins fuse cells via an universal intermediate in which the outer leaflets of the plasma membranes merge before the inner leaflets (hemifusion). The FF proteins are genuine cell fusogens because they are: (1) Essential for ectodermal and mesodermal cell fusion. (2) Expressed specifically on the surface of both fusing plasma membranes at the time of fusion. (3) Sufficient to fuse cells that normally do not fuse *In Vivo* and in tissue culture cells. We will also describe how these fusogens sculpt epithelial toroidal cells, muscular rings, long tubes, and complex neuronal trees containing repetitive menorah units. Understanding the mechanisms of cell fusion and membrane sculpting by EFF-1 and AFF-1 may facilitate the identification of other fusogens required for fertilization and somatic fusion in different organisms.

Clathrin-mediated endocytosis (CME) is the major mechanism of cargo internalization from the plasma membrane (PM). CME initiates as a result of recruitment of adaptors and clathrin to create a small invaginating region of the PM termed a clathrin-coated pit (CCP). Following a maturation process involving recruitment of other endocytic accessory factors, CCPs pinch off and form clathrin-coated vesicles. The spatial and temporal hierarchy of molecular events governing CCP maturation is not yet fully defined. Several CCP factors bind phosphatidylinositol-(4,5)-bisphosphate (PIP2) and PIP2 is required for CCP initiation. However, whether PIP2 is synthesized or enriched within CCPs, and how this might participate in CCP maturation remains unknown. To determine how PIP2 dynamics may control CCP maturation we have applied a combination of time-lapse TIRF microscopy of cells expressing fluorescently-labeled clathrin with computational analysis of CCP dynamics. Monitoring lifetimes allows identification of 3 distinct subpopulations of CCPs: 2 short-lived abortive and a long-lived productive subpopulations; we previously proposed that progression to the productive CCP stage is gated by an endocytosis restriction/checkpoint. Using this method in combination with siRNA silencing and overexpression, we find that PIP2 synthesis by phosphatidylinositol-5-kinase type I (PI5K1) positively controls CCP initiation and aids in the efficiency of productive CCP maturation. PIP2 degradation by the phosphatases OCRL1 and synaptojanin1 (SJ1) negatively regulates the efficiency of progression of CCPs to the productive stage and also increase the rate of turnover of abortive CCPs. In contrast, synaptojanin2 (SJ2) is required for efficient progression past the endocytic restriction/checkpoint to the productive CCP stage. SJ1,SJ2 and OCRL, but not PI5K, localize to CCPs. Taken together, these results suggest a model in which PIP2 is produced in the bulk PM and aids in recruitment of endocytic proteins. SJ2 acts early in CCP maturation, likely aiding CCP growth by ensuring dynamic turnover of PIP2 within nascent CCPs. SJ1 and OCRL negatively regulate CCP maturation and may provide input into the endocytic restriction/checkpoint machinery.
Regulation of Phosphoinositide Dynamics at Endocytic Clathrin-Coated Pits by the Inositol 5-Phosphatase Ship2.

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Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] and its phosphorylated product phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P3] are two major phosphoinositides (PIs) primarily concentrated at the plasma membrane where their levels are tightly controlled in space and time by kinases, phospholipases and phosphatases. Endocytosis is tightly coupled to the dephosphorylation of these PIs and we have previously identified three inositol 5-phosphatases involved in such dephosphorylation: synaptojanin 1 and 2 as well as OCRL. Here we show that the inositol 5-phosphatase SHIP2, a negative regulator of PI(3,4,5)P3-dependent insulin signaling, is also concentrated at clathrin-coated pits (CCPs) and regulates their dynamics. As shown by TIRF microscopy, SHIP2 is recruited to early stage CCPs and disappears from them before fission. Recruitment of SHIP2 to CCPs is mediated by intersectin 1. SHIP2 knockdown and the acute inducible recruitment to the plasma membrane of a PI 3-kinase, i.e. two conditions expected to increase PI(3,4,5)P3 levels, shorten CCP lifetime. Both localization at CCPs and phosphatase activity of SHIP2 are required for efficient negative regulation of insulin signaling. These findings corroborate the concept that phosphoinositide metabolism plays a key role both in endocytosis and in signaling at endocytic CCPs.

Characterization of Atlastin-Mediated Membrane Fusion In Vitro.

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Homotypic membrane fusion is essential for the formation and maintenance of the dynamic structure of the endoplasmic reticulum (ER). The large GTPase atlastin has recently been shown to be important in ER formation and maintenance and is able to promote the tethering and homotypic fusion of liposomes in vitro. Atlastin, along with mitofusins, represents a novel class of fusogens distinct from SNARE's and viral fusogens. Our current studies are aimed at determining the mechanism by which Drosophila atlastin tethers distinct membranes and drives the merging of lipid bilayers. We are conducting a structure-function analysis of atlastin domains required for membrane fusion and oligomerization. We have found that carboxy-terminal truncations result in an impaired ability to fuse membranes. Additionally, mutations designed to disrupt a predicted juxtamembrane coiled-coil domain reduce GTPase activity and prevent the ability of atlastin to fuse liposomes. Furthermore, acute addition of the amino-terminal cytoplasmic domain of atlastin inhibits the fusion of atlastin proteoliposomes while the cytoplasmic domain lacking the predicted coiled-coil domain has no effect. These results suggest that the carboxy-terminal 70 residues are important for atlastin function and that atlastin may form homo-oligomers through a juxtamembrane coiled-coil to promote homotypic membrane fusion.

A Combined Genetics and Lipidomics Approach to Explore Metabolism and Functions of Membrane Lipids.

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With the burgeoning appreciation of the critical functions of lipids in biological processes, and aided by advances in technologies that afford an '-omic-centric' view of the lipid inventory of biological systems, the field of lipidomics has emerged over the last 5 to 10 years. In particular, the development of electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation
(MALDI) mass spectrometry (MS) has led to the realisation of a new level of sensitivity, resolution and throughput for simultaneous analysis of multicomponent lipid mixtures, generating a metabolic fingerprint containing quantitative as well as qualitative information on the system investigated. We previously developed a novel label-free approach, based on ESI-MS and chemometry, to detect changes in the metabolic fingerprint during a cellular perturbation. Without the need for prior knowledge of the chemistry of the analytes, the tool is particularly powerful in detecting changes that cannot be easily anticipated. The availability of a wide range of genetic and chemogenetic libraries for various model organisms, including the budding yeast Saccharomyces cerevisiae and the fruit fly Drosophila melanogaster, offers unparalleled opportunities for genome-wide screens to discover and/or characterise mutants involved in lipid metabolism, transport and turnover, as well as the revelation of novel lipid functions. For the first time, we combined lipidomics with yeast and fly genetics to probe for novel enzymatic activities and modulators of known ones, with a focus on sphingolipids. We further provided evidence of an exquisite specificity of interactions between lipids in living cells that is critical for cellular functions.

**Minisymposium 13: Nuclear Structure (819 – 824)**

**819**

**Nuclear Body Crosstalk.**

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Cajal bodies (CBs) are conserved nuclear subdomains that are present in both animal and plant cells. CBs contain high concentrations of the marker proteins coilin and SMN, as well as the spliceosomal small nuclear ribonucleoproteins (snRNPs) U1, U2, U4 and U5. In metazoa, related structures known as histone locus bodies (HLBs) are found located adjacent to the replication-dependent histone gene cluster. HLBs are critical for efficient histone mRNA formation *in vivo*, and contain factors required for histone gene transcription and pre-mRNA 3’ end formation, including NPAT, FLASH and the U7 snRNP. HLBs and CBs are often found in close proximity to each other in human cancer cell lines and *Drosophila* nurse cells, but whether this represents a functional association is not known. Here we present a study of the interconnectivity of *Drosophila* CBs and HLBs by utilizing mutants in the snRNP and histone mRNA biogenesis pathways. Intriguingly, in a variety of both snRNP and histone biogenesis mutants, CBs are disrupted and coilin is delocalized, forming a kind of ‘cloud’ around the nurse cell HLBs. This phenotype was extremely penetrant. Notably, HLBs remained largely unaffected in the majority of these backgrounds. The strikingly similar phenotypes in each of these classes of mutation suggest that CB integrity is dependent on ongoing histone pre-mRNA processing. Other examples of molecular crosstalk between CBs and HLBs in both wildtype and mutant backgrounds will be discussed.

**820**

**Nuclear Protein and Cajal Body-Associated Coilin Modulates the Mitochondrial Apoptotic Pathway Following UV-C-Induced Chromatin Damage.**

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Coilin is a nucleoplasmic protein and a major component of the nuclear domains called Cajal bodies (CBs). Since its description in the early 1990s no clear biological activity has been assigned to the protein. Coilin is highly dynamic and its nuclear distribution changes dramatically following various stressful stimuli such as irradiations, drugs, and even viral infection. We recently described the accumulation of coilin at, and its association with centromeres following centromeric chromatin damage induced by the ICP0 protein of herpes simplex virus type 1 or by siRNA depletion of centromere protein CENP-B. We hypothesized that coilin might participate in a cell response dedicated to sense and signal aberrant chromatin structures. We also assumed
that any stress that would abruptly affect chromatin might involve coilin in a signalling pathway whatever the chromatin is centromeric or not. UV-C are known to profoundly modify the chromatin structure and to induce apoptosis in a p53-independent manner. We therefore tested the contribution of coilin in UV-C-induced apoptosis. Coilin was depleted using siRNAs in various cell lines before they were stressed with UV-C. The degree of susceptibility to apoptosis was dose- and cell-type dependent but the lack of coilin systematically reduced apoptotic marks such as chromatin condensation, phosphatidylserine externalisation, activation of effector caspases and inactivation of PARP-1. We showed using various apoptosis-inducing stress, including UV-C, etoposide and TRAIL ligand, that the mitochondrial-controlled pathway was suppressed in coilin-depleted HeLa cells. This lack of susceptibility to apoptosis was correlated with a defect in cytochrome c release out of the mitochondria without significantly affecting the amount of pro- and anti-apoptotic proteins. We then found that Bax oligomer formation was dramatically reduced. Altogether, this study describes the role of coilin as a protein that acts as a modulator of the apoptotic intrinsic pathway following a stress that affects the chromatin structure.

821
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Nuclear speckles provide important spatial organization and dynamic regulation for pre-mRNA processing factors in mammalian cells. While the nuclear speckle proteome is complex, little is known at the molecular level about how these factors are organized into nuclear speckles or how alterations in the organization of these factors impacts gene expression. We have discovered a new function for a large (2564 amino acid) nuclear speckle protein called Son in maintaining the organization of pre-mRNA processing factors in nuclear speckles. Depletion of Son by RNAi causes snRNP and serine-arginine rich (SR protein) splicing factors to undergo dramatic disorganization into doughnut-shaped nuclear speckles. Rescue of the disorganized nuclear speckle phenotype requires a region of Son with multiple tandem repeat motifs that are unique to Son. This demonstrates that the tandem repeats of Son are necessary for appropriate localization of pre-mRNA processing factors, and it suggests a potential role for Son as a nuclear speckle scaffold. Son depletion does not alter protein levels of other splicing factors, and it does not reduce global transcription or constitutive splicing. However, Son depletion can affect alternative splice site selection. Surprisingly, in addition to its nuclear functions, Son depletion also results in decreased cell proliferation due to growth arrest in mitosis. Son is critical for promoting the transition from metaphase to anaphase. Son is therefore essential for nuclear organization and function, as well as for normal cell cycle progression.

822
An Atypical Progeria Mutation Alters Lamin Polymerization, Nuclear Assembly and Chromosome Organization.
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Numerous mutations in the human nuclear lamin a gene (LMNA) cause the premature aging disease, progeria. The most common form of progeria produces LAΔ50/progerin, a truncation near the C-terminus of lamin a (LA) leading to its permanent farnesylation and abnormal nuclear membrane association. There are also several mutations located in the α-helical central rod domain of LA which would not impact its state of farnesylation. However, this domain is required for the normal polymerization of LA into higher order structures. We studied cells from a patient...
with one of these mutations, E145K, in which the nuclei are severely lobulated with abnormal A- and B-type lamin networks, clustered centromeres, mislocalized telomeres and disorganized pericentric heterochromatin. Expression of FLAG-E145K-LA induces similar changes in HeLa cells. Live cell imaging of these transfected cells shows that lobulation originates as nuclei assemble in daughter cells and that the clustered centromeres normally seen in early G1 are abnormally retained throughout interphase. This abnormal positioning of chromosomes results in the mislocalization of DNA replication factories which is correlated with premature replicative senescence in patient cells. In Vitro analyses of E145K-LA polymerization by ultracentrifugation, negative staining and cryo-electron tomography show severe defects in the assembly of lamins into higher order structures. Similar assays of LAΔ50/progerin show that all steps in the polymerization process are normal. The results demonstrate that the E145K mutation impacts lamin assembly and nuclear architecture differently than the LAΔ50/progerin mutation. The results also shed new light on the wide array of lamin functions in the nucleus. Supported by NIA and a Ellison Medical Foundation Senior Scholar Award (RDG), EURO-Laminopathies project of the European Commission (HH), German-Israel Foundation (HH and OM), Fritz-Thysen Stiftung (OM), Sigrid Juselius Foundation (PT) and Deutsche Forschungsgemeinschaft (KP).

823
Integrity of the Nuclear Lamina Is Required for Germline Stem Cell Homoestasis.
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The nuclear lamina is a filamentous network that underlines the nuclear envelope. Lamina components include lamins, as well as the LEM domain (LEM-D) proteins, named for LAP2, emerin and MAN1. LEM-D proteins establish a bridge between the nuclear lamina and chromatin through the ~40 amino acid LEM domain (LEM-D) that binds Barrier-to-Autointegration Factor (BAF), a DNA and histone binding protein. Mutations in genes encoding LEM-D proteins cause tissue-restricted human disease, such as Emery-Dreifuss muscular dystrophy, even though these genes are globally expressed. We are studying a Drosophila emerin homologue called Otefin. Genetic analyses demonstrate that loss of Otefin causes tissue-restricted defects that are limited to female sterility. Mutant females contain rudimentary ovaries indicative of an early block in oogenesis. In a wild type ovary, germline stem cells (GSCs) divide asymmetrically to form cystoblasts that differentiate into oocytes. BMP signaling plays a central role in controlling GSC self-renewal, establishing a limiting signal that represses expression of differentiation genes in GSCs, but not in the daughter cystoblasts. BMP signals emanate from the adjacent somatic cap cells that comprise the instructive cells of the specialized GSC niche. Immunohistochemical analyses show that loss of Otefin causes excessive BMP signaling. We find that terminal filament cells lacking Otefin adopt a cell fate similar to the instructive cap cells, which may account for the elevated BMP response. These effects result in changes of expression in BMP responsive genes, thereby blocking cystoblast differentiation. In addition, otefin defective germ cells display lobulated and thickened nuclear lamina, which may contribute to altered nuclear functions. Within ten days, otefin females show loss of cells in the somatic GSC niche, as well as of germ cells, representing an accelerated aging phenotype. These data demonstrate that Otefin plays an essential role in GSC maintenance through regulation of differentiation of the somatic cells of the niche and BMP signaling. Our findings support models suggesting that laminopathies arise from dysfunction of the homeostasis in stem cell populations.

824
Distinct Clustering of Active and Repressed Genes at the Nuclear Periphery of Mouse Embryonic Stem Cells.
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A gene’s location in the nucleus can impact its expression. A gene can be influenced by another nearby gene and by association with a sub-nuclear compartment, such as the nuclear periphery. However, the mechanisms mediating gene 3D clustering or associations with nuclear compartments are poorly understood. We previously discovered that the set of genes across a 5
Mb region of mouse chromosome 14 cluster together in the nucleus to form 3D gene hubs. These hubs form at or near the nuclear periphery in multiple cell types, including mouse embryonic stem cells (ESCs). To determine mechanisms mediating this 3D gene clustering, we investigated the relative locations of these genes based on their activity and epigenetic state. In ESCs, four of the genes are in a repressed and epigenetically “bivalent” state. Bivalent genes are marked by covalent histone modifications indicative of both transcriptionally active (histone H3 K4-trimethyl) and repressed (histone H3 K27-trimethyl) chromatin. The locations of the repressed genes in ESCs were compared to four active genes in the same chromosomal region. We found that the repressed genes form homotypic clusters within the nucleus, as do the active genes. These two types of genes clusters are adjacent to each other but non-intermingling. In addition, the repressed genes on average localize closer to the nuclear periphery than the active genes, although active genes do co-localize with the lamina in some cells. Interestingly, loss of the histone H3 K27-trimethyl mark from the repressed genes due to mutation of the Polycomb group gene Eed does not alter gene association with the nuclear periphery. However, the 3D organization of these normally repressed genes is altered relative to the active gene set. Together, our findings suggest that gene 3D clustering is related to transcriptional activity and epigenetic state. Furthermore, these data indicate that trimethylation of H3K27 is not required for localization to the nuclear periphery, but rather that this epigenetic mark plays a role in the relative arrangement of genes at the nuclear periphery.

Minisymposium 14: Organization and Dynamics of the Cytoskeleton (825 – 830)

825 Intermediate Filaments Bind Tubulin and Function as a Local Tubulin Reservoir to Modulate the Assembly of Microtubules.

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Neurofilaments (NF) assemble from three intermediate-filament proteins, are exceptionally stable and contribute to the radial growth of axons. Microtubules (MT) are dynamic structures that assemble from tubulin dimers to support intracellular transport of molecules and organelles. Here we show that intermediate filament proteins, including NF, desmin, GFAP, keratin, and vimentin, contain motifs in their N-terminal domains that bind unassembled tubulin. Peptides corresponding to these Tubulin-Binding-Sites inhibit MT polymerization in vitro. Some are taken up by cultured cells where they disrupt MT and cause an arrest of cell division. In transgenic mice in which NF are aggregated in cell bodies and thus withheld from the axonal compartment, the levels of tubulin accumulated in both cell bodies and axons is normal. However, in their cell bodies, MT are not detectable while they assemble in excessive numbers in axons. These observations indicate that NF and other intermediate filaments can function as a dynamic tubulin reservoir modulating local MT assembly. This property places them in a unique position to contribute to a wide array of MT-dependent cell functions. This work was supported by Association Francaise contre les Myopathies, Association pour la Recherche sur le Cancer, Fonds European de Developement Regional, and Institut National du Cancer to J. Eyer, the German National Genome Research Net to R. Frank and the Canadian Institutes of Health Research to A. Peterson. A. Bocquet and R. Berges were supported by Angers Agglomeration and ARC.

826 Acetylation of Microtubules Is a Regulator of Their Sensitivity to Severing by Katanin in Neurons and Fibroblasts.

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Here we investigated whether the sensitivity of microtubules to severing by katanin is regulated by acetylation of the microtubules. During interphase, fibroblasts display long microtubules with discrete regions rich in acetylated tubulin. Overexpression of katanin for short periods of time produced breaks preferentially in these regions. In fibroblasts with experimentally enhanced or diminished microtubule acetylation, the sensitivity of the microtubules to severing by katanin was increased or decreased respectively. In neurons, microtubules are notably more acetylated in axons than in dendrites. Experimental manipulation of microtubule acetylation in neurons yielded similar results on dendrites as observed on fibroblasts. However, under these experimental conditions, axonal microtubules were not appreciably altered with regard to their sensitivity to katanin. We hypothesized that this may be due to the effects of tau on the axonal microtubules, and this was validated by studies in which overexpression of tau caused microtubules in dendrites and fibroblasts to be more resistant to severing by katanin in a manner that was not dependent upon the acetylation state of the microtubules. Interestingly, none of these various findings apply to spastin, as the severing of microtubules by spastin does not appear to be strongly influenced either by the acetylation state of the microtubules or by tau. We conclude that sensitivity to microtubule-severing by katanin is regulated by a balance of factors including the acetylation state of the microtubules and the binding of tau to the microtubules. In the neuron, this contributes to regional differences in the microtubule arrays of axons and dendrites.

827
Microtubule Organization and Motor Co-Operation in Long-Range Endosomes Motility.
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In elongated cells of the model fungus Ustilago maydis microtubules serve as tracks for long-range motility of membranous cargo. The plus-ends of microtubules are directed towards the growth region, whereas the minus-ends are located at mobile and bi-polar nucleation sites. The position of these microtubule organisation centres depends on the activity of cytoplasmic dynein, which results in a complex anti-polar microtubule array. Early endosomes utilise this array for long-range motility, which is mediated by kinesin-3 and dynein. Both motors move their cargo in opposite direction, and their activity is therefore determined by the polarisation of the microtubule array. Live cell imaging of photo-activated early endosomes and the respective motors suggest a cooperative mechanism by which counteracting motors cope with the anti-polar microtubule orientation in order to govern long-range motility of early endosomes.

828
A Network of Formin Inhibitors Required for Actin Assembly and Architecture.
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Formins are a conserved family of actin nucleation- and elongation-promoting factors with essential roles in governing eukaryotic cell shape and behavior. In vivo, the potent activities of formins must be tightly controlled in a temporal manner to yield actin filaments of the appropriate length and thereby maintain actin network architecture and function. In many cases, the timing of formin activation appears to be under the control of Rho proteins; however, mechanisms for inhibiting and/or inactivating formins are only just beginning to emerge. Recently, we identified Bud14 as a strong inhibitor of the yeast formin Bnr1 that can displace the Bnr1 FH2 domain from rapidly growing barbed ends of filaments (Chesarone et al., 2009). Further, deletion of BUD14 led to abnormalities in actin cable architecture and secretory vesicle transport. Here, we identify the orphan kinesin Smy1 (suppressor of myo2) as a novel direct inhibitor of Bnr1. Like Bud14, purified Smy1 binds to the FH2 domain and potently suppresses the actin assembly activity of Bnr1. A specific region of Smy1 outside of its putative motor domain mediates these effects. Further, smy1Δ and bud14Δ show synthetic defects in cell growth and compounded defects in actin cable assembly, suggesting that they coordinate the timing of Bnr1 activity in vivo. Both Smy1 and Bud14 depend on Myo2-dependent transport on actin cables for their delivery to polarity sites where formins localize, suggesting a possible negative feedback loop that
attenuates Bnr1 activity. Interestingly however, Smy1 did not displace Bnr1 from growing barbed ends, suggesting that it has a mechanistically distinct role from Bud14 in inhibiting Bnr1. Together, this work demonstrates that even a relatively simple eukaryote like budding yeast requires multiple formin binding partners to regulate the precise timing and duration of formin activity, and thus maintain proper actin network organization and function.

829
Regulation of Rho Proteins Homeostasis by RhoGDI1.
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Rho GTPases are molecular switches that regulate many cellular functions including cell migration, organization of the actin cytoskeleton and adhesion dynamics. At steady state, most Rho proteins are bound in the cytosol to Rho Guanine nucleotide Dissociation Inhibitors (RhoGDI). The major GDI in mammalian cells, RhoGDI1 binds to RhoA, Rac1 and Cdc42 and is expressed at a level approximately equal to the sum of these Rho family members. RhoGDI1 has generally been considered to passively hold Rho proteins in an inactive state within the cytoplasm. However, the precise biological function of RhoGDIs has remained elusive. Here we show that RhoGDI1 controls the homeostasis of Rho proteins in eukaryotic cells. We found that RhoGDI1 depletion in mammalian cells, as well as in S. cerevisiae, triggers an almost complete degradation of the major Rho GTPases. In the absence of RhoGDI1, cytosolic Rho GTPases that have been geranyl-geranylated are cleared from the cytosol by the proteasome. Since the level of RhoGDI1 is limiting, there is competition between different Rho proteins for binding to RhoGDI1. We show that overexpression of an exogenous Rho GTPase leads to the displacement of endogenous Rho proteins from RhoGDI1 and their subsequent degradation. These results demonstrate an unanticipated crosstalk between Rho GTPases mediated by binding to RhoGDI1. The competition of Rho proteins for binding to RhoGDI1 raises important questions about some of the conclusions drawn from studies that manipulate Rho protein levels. In many cases the responses of cells may arise not simply from the overexpression or knockdown per se, but from additional effects on the levels of other Rho GTPases due to competition for binding to RhoGDI1.

830
The Arp2/3 Activator WASH Controls the Fission of Endosomes through a Large Multiprotein Complex.
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The Arp2/3 complex is an actin nucleator that generates branched actin networks. It is activated by Nucleation Promoting Factors (NPFs) such as N-WASP, Scar/WAVE, WHAMM or JMY. Recently, the WASH family of NPFs has been identified but its cellular role is unclear. Here we show that endogenous WASH localizes to a microdomain of sorting and recycling endosomes. Accordingly, WASH is required to generate actin networks associated with these endosomes. NPF regulation is provided by multiprotein complexes, as exemplified by Scar/WAVE. We purified a novel stable multiprotein complex containing seven subunits, including WASH and the heterodimer of capping protein (CP). In agreement with cellular observations, the purified WASH complex activates Arp2/3 mediated actin nucleation and binds directly to liposomes. Interestingly, the WASH complex also has a low capping activity, suggesting a tight control of CP within the complex. Specific inhibition of WASH mediated actin polymerization, obtained by microinjection of blocking antibodies or through siRNA mediated depletion, induces the formation of long membrane tubules pulled out from endosomes along microtubules, suggesting a role of WASH in the fission of transport intermediates. Indeed, we found that WASH interacts with dynamin and that inhibition of dynamin phenocopies WASH depletion. In line with such a role in endosome fission, WASH is required for efficient transferrin recycling. Together these data suggest that the
WASH molecular machine, integrating CP with a NPF in an unprecedented manner, controls the fission of endosomes through an interplay between the forces generated by microtubule motors and actin polymerization.

Minisymposium 15: Systems Biology (831 – 836)

831
Sake to Chocolate: Systems Genetics and Natural Variation in Yeast.
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Leveraging the information in individual genome sequences is of increasing relevance to understanding human health and is essential to the development of predictive, preventive, and personalized medicine. Common diseases such as asthma, diabetes, heart disease are complex traits, with interactions that can range from simple binary relationships to many genes with complicated interdependencies. Genetics, the study of how differences in DNA sequence affect the observable characteristics of an individual, is fundamentally a systems science. Systems genetics uses large-scale, high-throughput methods to decipher the network of gene functions and interactions in an organism. This network, in which genes interact with each other and with the environment in complex ways, provides the framework through which biological information is transmitted, integrated, and ultimately used by the downstream networks of proteins, RNAs, and small molecules. Here we present an example of how systems level approaches can be used to dissect a complex quantitative trait, the “fluffy” colony morphology phenotype. We start with a collection of natural isolates of Saccharomyces cerevisiae from diverse sources (e.g. laboratory strains, clinical isolates, sake brewing, soil samples) and geographic locations (six continents) as a rich source of naturally occurring genetic polymorphisms. We mate these parental strains, isolate large numbers of recombinant progeny, assay their phenotypes across numerous conditions, and genotype them by multiplexed, next generation DNA sequencing. Our results demonstrate the power of integrating automated image analysis, comprehensive molecular network data (including RNA expression and mass spec based proteomics), spatial and temporal information, and large-scale genetic analysis. We believe such approaches will increase our understanding of the underlying structure of complex traits and lead to methods for predicting phenotypes in more complex systems, such as humans.

832
A High-Content Screen to Functionally Classify Proteins Required for Cell Viability and Division.
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Systems biology analyzes molecular assemblies within functional networks. The success of this approach relies upon available systematic and functional information for the components of these molecular assemblies. RNA-mediated interference (RNAi) is a key method for linking genes to their cellular functions. In addition to relatively ‘low content’ single reporter assays, RNAi has been used to perform high-content microscopy-based assays, integrating spatial and/or temporal information. This approach has been used to characterize the set of genes required for cell viability and division in C. elegans, a model system for molecular analysis of animal cell division. Of the 20,000 genes, 10% are required for embryo production or viability; these genes were previously screened by filming the first two embryonic divisions following RNAi depletion. This screen allowed for the functional classification of ~400 genes, however it did not provide high quality functional information on the ~560 genes whose inhibition blocks embryo production (the sterile collection). Here, we functionally characterize the sterile collection by performing a second
high-content screen, using two-color fluorescence confocal microscopy to examine gonad structure, in living worms, after individual depletion of the 560 sterile gene products. Gonad-morphology data was analyzed by binary scoring for 94 potential defects, and a clustering algorithm was used to group genes with similar phenotypic profiles. The clusters were then re-examined by eye for accuracy. Our analysis placed genes into ~20 broad groups that could be partitioned into ~100 different phenotypic classes, which typically corresponded to subunits of specific protein complexes. As a result, we have classified the ~170 previously "unknown" genes into functional groups and have provided additional data on the ~390 "known" genes. Upon publication, our data will be pooled with the previous high-content screen data and collectively housed in the PhenoBank online database, creating a valuable resource for the scientific community. This work provides an important platform for systems biology based analysis of the pathways contributing to embryo production, cell division, and development.

833 Mitochondrial Network Morphology: Control of Abundance and Topology in Budding Yeast.
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We want to understand the fundamental unsolved questions of how the 3-dimensional geometry of cellular structures is generated and how this geometry contributes to cellular functions. An ideal system to approach this question is the dynamic, physical network of interconnected tubules that makes up mitochondria. In budding yeast the mitochondrial network localizes at the cell periphery, undergoes constant fusion and fission dynamics that remodel the network, and exhibits consistent changes in morphology under different physiological conditions. We have developed a method to quantify the 3D mitochondrial morphology by considering the mitochondria as a network of edges (the tubules) and nodes (the branch points where tubules connect). Live mitochondria are rapidly imaged in 3D at high resolution using Spinning-disk Confocal microscopy. Specialized segmentation and 3D skeletonization methods are applied to the image stacks to extract the mathematical graph of the mitochondrial network. for validation and testing the limits imposed by microscopy imaging, we create theoretical microscope images from predetermined skeletonized structures and compare these to the 3D skeletons obtained by our methodology. We then apply concepts and methods from complex networks and shape analysis research to obtain measurements of the total amount, arc lengths, density, and connectivity of mitochondrial tubules in the cell. Using our methodology, we investigate mechanisms of mitochondrial abundance control during cell cycle progression. During budding, we find that mitochondria accumulate in the bud at a constant rate independent of the volume or density of mitochondria in the mother. Differences in mitochondrial density between individual cells or different growth conditions can be explained by differences in the cell growth rate and the duration of the cell cycle. We also analyze how topological network properties depend on tubule density at the cell surface and on fusion and fission dynamics. We find that the frequency of fusion/fission events is directly correlated with, and may be regulated by, the amount of mitochondria in the cell.

834 Quantitative Output of a Signaling Pathway Is Controlled by Microtubule-Mediated Repositioning of the Nucleus.
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Genetically identical cells differ from each other in size, shape and other aspects. Such heterogeneity can affect the quantitative outputs of cell signaling systems; thus, mechanisms that
adjust transmitted signal to account for individual variability could be advantageous. We showed earlier that the mating pheromone response system of S. cerevisiae compensates for cell-to-cell variation by scaling the amplitude of the transmitted signal (Nature, 2005. 437 (7059) 699). Here, we designed a genetic screen to identify components responsible for controlling cell-to-cell variation in transmitted signal amplitude. We found that deletion of a class of genes increased variability in pheromone-responsive gene induction assayed at the single cell level, but did not affect the population’s average pathway output. This class included BIM1 and KAR3, whose products attach to and control the length of microtubule-mediated links between the nucleus and the specialized plasma membrane site where pheromone receptors cluster. We therefore examined the sub-cellular position of the nucleus in these mutants. In time course experiments, the position of the nucleus in wild-type cells exposed to pheromone was stable and pheromone-dependent: the higher the dose, the further the nucleus was from the signaling site. By contrast, in both mutants, the nucleus moved randomly within the cell and its average distance to the signaling site did not correlate with pheromone dose. Thus, mutations that increased cell-to-cell variation in transmitted signal amplitude also increased variability in the position of the nucleus. Perturbing the connection between the nucleus and the signaling site impaired the ability of cells to select the mating partner producing the most pheromone. Thus, our results revealed that a mechanism that controls the position of the nucleus enhances the precision of a signaling system, perhaps by enabling the cell to adjust the amplitude of the transmitted signal to that cell’s shape and size. This mechanism might be operational during response to developmental cues, when cells of diverse size and shape must manifest a uniform output to a level of signal presented to them at a specific position within a gradient.

835
Reconstructing the EGFR Autocrine Signaling Network by Integrating Proteomic and Genomic Data.
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Many types of cells integrate information from multiple signaling pathways by modulating EGFR autocrine signaling - a process known as EGF receptor transactivation. To better understand the mechanism(s) by which signals from different receptors are integrated by cells, we created a multi-tier model of the major signaling pathways in human mammary epithelial cells. We first defined the topology of these pathways by using a combination of quantitative MS-based proteomics data and gene expression profiles together with curated protein interaction data. We then experimentally treated the cells with a variety of growth factors followed by global phosphoproteomics analyses. The pattern of protein phosphorylation was then overlaid on the reconstructed network topology to generate hypotheses regarding how different pathways interact to generate a particular response, such as ERK phosphorylation. Some of these hypotheses were then tested by targeted experiments and by steady-state perturbation of the autocrine signaling network. This combined “top-down” and “bottom-up” approach for reconstructing signaling networks generated some intriguing findings regarding their architecture. First, there are numerous feedback loops, both positive and negative, that operate over a wide range of different temporal scales and respond to different levels of stimulation. Second, many different adaptor proteins are shared between multiple signaling pathways, but are expressed at low levels relative to receptors. Modeling suggests that this differential expression can give rise to a signaling hierarchy between different pathways based on both receptor-ligand abundance and affinity for adaptor proteins. Finally, we found that signal integration occurs separately at the level of individual pathway activation and at the level of gene transcription. Our results suggest that both quantitative proteomics and gene expression data can greatly facilitate the reconstruction of signaling networks by imposing realistic constraints on mathematical models of these networks and by revealing multiple levels of regulation.
836
Cell to Cell Variability in the Responses of Mammalian Cells to Death Ligands.
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Cell to cell variability in phenotype and fate is important for tissue homeostasis, the emergence of drug resistance and the evolution of tumors. I will discuss cell to cell variability in the responses of clonal populations of human cells to TRAIL, a prototypical inducer of receptor-mediated (extrinsic) apoptosis and an investigational therapeutic. Some TRAIL-treated human cells die within ~40 min, others only after 12 hr and some live indefinitely. During this variable delay initiator caspases (ICs) are active in processing substrates such as Bid but mitochondrial membrane permeabilization (MOMP) does not occur and downstream effector caspases (ECs) are catalytically inactive. We have tested three possibilities to explain why some cells in a notionally uniform population die rapidly and others slowly or not at all (i) cell to cell genetic or epigenetic variation (ii) the involvement of one or more involving biochemical processes subject to stochastic fluctuation (iii) difference in cell state such as. position in the cell cycle or protein concentrations. To distinguish these three possibilities we compared the fates of sister cells exposed to TRAIL using live-cell microscopy. Genetic and epigenetic factors are transmitted with near-perfect fidelity; reaction rates dominated by stochastic fluctuations are more similar in sisters than in any two cells chosen at random and variability arising from differences in mRNA or protein levels should be transiently heritable (because division is a binomial process in which sister cells inherit roughly equal numbers of abundant cytosolic and nuclear factors but drift towards the population average over time). The later explanation is correct - natural fluctuations in protein levels dominate phenotypic heterogeneity in extrinsic apoptosis and cause transient phenotypic heritability. Variability can be manipulated by protein over-expression and drugs and I will describe analytic methods for modeling these perturbations. Finally, I will show that natural variation can be sufficient to cause some cells to follow one biochemical pathway to death and another set of genetically identical cells to follow a fundamentally different pathway.

Symposium 3 - All You Can Be - The Biology of Multipotency (837 – 839)

837
Epigenetic Regulation of Stem Cell Fate in Plants.

Plant stem cells are located in specialized niches, termed meristems, at the growing tips of the plant. Stem cell fate within the shoot apical meristem (SAM) is specified in part by the KNOX homeobox genes. Down-regulation of KNOX expression is a key factor that distinguishes stem cells and their immediate indeterminate derivatives in the SAM from lateral organ initials. Moreover, normal leaf development requires the continued silencing of these stem cell regulators. We have shown that this process is mediated by the DNA-binding proteins ASYMMETRIC LEAVES1 (AS1) and AS2, which target the chromatin-remodeling factor HIRA to the KNOX loci in differentiating lateral organs. More recently we have shown that HIRA facilitates the recruitment of Polycomb Repressive Complex2 to the KNOX loci, which catalyses the establishment of the H3K27me3 repressive mark. This mark is recognized by LIKE-HETEROCHROMATIN PROTEIN1, which through a currently unknown mechanism, leads to a stable repressive chromatin state at the KNOX loci that allows cellular differentiation. Our research has shown that cellular differentiation is achieved via an epigenetic mechanism in which HIRA serves as an intermediary factor that recruits PRCs to pluripotency determinants. A role for HIRA and PRC2 in regulating stem cell homeostasis is conserved in animals, presenting the possibility that our results in plants uncover a highly conserved epigenetic mechanism that allows cellular differentiation.
**838**

**Regulation of Stem Cell Function in Aging Organisms.**

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Aging of multicellular organisms typically involves a progressive decline in normal cell replacement and repair processes, resulting in a number of physiologic deficiencies, including inefficient muscle repair, reduced bone mass, and dysregulation of blood formation (hematopoiesis). While defects in tissue-resident stem cells clearly contribute to these phenotypes of aging, it has been unclear to what extent these defects arise from stem cell intrinsic alterations versus age-related changes in the stem cell supportive microenvironment, or niche. To address this important question, we have established complementary In Vivo and In Vitro heterochronic models to assess stem cell and niche cell activity with age. Our data indicate that stem cell-supportive niche cells become deregulated with age, and this deregulation contributes directly to hematopoietic stem cell (HSC) dysfunction. Furthermore, we find that age-dependent defects in both niche cells and HSCs are controlled systemically, and can be reversed by exposure to a young circulation. These studies reveal a novel and critical role for both local and systemic factors in signaling age-related changes in HSC engraftment activity and lineage potential, and highlight a new paradigm in which blood-borne factors in aged animals act via local niche cells to induce age-dependent disruption of stem cell function. Modulation of stem cell interactions with the niche thus represents a promising avenue for restoring youthful function in aging tissues.

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**839**

**Germ Cells Are Forever.**

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From generation to generation, germcells provide the continuity of life. Despite their critical role, we still know little about how germcells are initially specified and what allows germcells to evade the deadly fate of the soma. Germcells are highly specialized; their role is to produce gametes and to escape somatic differentiation. But, in contrast to somatic cell fates, no master-regulator transcription factor has been identified that specifies germcell fate. from studies in flies, worms, and mice, three general features have emerged: 1) Transcriptional silencing coincides with germcells specification. 2) Conserved, germcell-specific translational regulators are active throughout the germcell life cycle. 3) Germ-line soma interactions are critical for different aspects of germcell behavior such as germcell migration, stem cell establishment and maintenance, protection of the germ line genome and differentiation of germcells into egg or sperm. We study germcell biology in Drosophila. We use genetic, biochemical and structural approaches to characterize critical germ plasm components essential for germcell fate specification. We find that germcells form by a specialized budding process, which is remarkably different from the process of somatic cell formation. Combining genetics with In Vivo imaging we have developed a dynamic picture of the migratory path of Drosophila germcells. Initiation of migration requires the Tre1 GPCR. GPCR activation mediates polarization, individualization and transepithelial migration of germcells. The lipid phosphate phosphatases (LPP) Wunen and Wunen 2, homologs of mammalian LPP3, guide germcells from the midgut to the somatic gonad and are also required in germcells for their survival and migration. Final, at the end of the migratory route germcells associate with the somatic gonad, HMGCoA reductase activity is required for the geranylation of a germcell attractant produced by the somatic gonad and exported by an unconventional secretory pathway.
**Symposium 4 - In a Pinch: Cell Division from Prokaryotes to Sex Cells (840 – 842)**

840

**Feedback Control of Mitosis.**

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Kinetochores (KTs) are proteinaceous structures implicated in the formation of load-bearing chromosome-microtubule attachments in mitosis. We are interested in the organization of KTs and in the feedback control mechanisms operating at the KT-microtubule interface. Two such control mechanisms exist: 1) An error correction mechanism protecting from incorrect KT-microtubule attachments. It allows the selective stabilization of correct attachments and the destabilization of incorrect attachments, thus promoting bi-orientation. 2) The spindle assembly checkpoint (SAC). It is required to synchronize the metaphase-anaphase transition with the completion of KT-microtubule attachment for all sister chromatid pairs in a cell. The relationship between error correction and the SAC is our main focus. To tackle this problem, we are taking an approach of biochemical reconstitution, structural analysis, and cellular studies. Both feedback control mechanisms operate near a large protein assembly, the Knl1-Mis12 complex-Ndc80 complex (KMN) network. The KMN network contains the main microtubule-binding activity of KTs. It is also crucial for recruiting the SAC components to KTs. Finally, it is a target of enzymes, such as the Aurora B and Mps1 kinases, that are essential for error correction. Thus, the KMN network may integrate adequate physical responses to the state of KT-microtubule attachment. I will present structural data on the KMN network, revealing the overall shape of the complex, as well as the molecular details of inter-subunit interactions that support complex oligomerization. I will also present our approach towards reconstitution of the reactions responsible for the recruitment of the SAC proteins to KTs and for error correction. Our efforts are based on a paradigm, strongly supported by studies with specific small-molecule inhibitors, that the Aurora B kinase is the upstream kinase in a hierarchical kinase cascade that controls error correction as well as the SAC response. Our working model is that the interaction of the Aurora B kinase with the KMN network is the crucial mechanism regulating the feedback mechanisms acting at KTs. A molecular model for how this might occur will be discussed.

841

**Structure of a Bacterial Dynamin-Like Protein Lipid Tube Provides a Mechanism for Self-Assembly and Membrane Curving.**

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Proteins of the dynamin superfamily mediate membrane fission, fusion and restructuring events by polymerising upon lipid bilayers and forcing regions of high curvature. In this work, we show the electron cryomicroscopy reconstruction of a bacterial dynamin-like protein (BDLP) helical filament decorating a lipid tube at ~11 Å resolution. We fitted the BDLP crystal structure and produced a molecular model for the entire filament. The BDLP GTPase domain dimerises and forms the tube surface, the GTPase effector domain (GED) mediates self-assembly, whilst the paddle region contacts the lipids and promotes curvature. Association of BDLP with GMPPNP and lipid induces radical large-scale conformational changes effecting polymerisation. Nucleotide hydrolysis seems therefore to be coupled to lipid dissociation and polymer disassembly rather than membrane restructuring. Observed structural similarities with rat dynamin 1 suggest our results have broad implication for other dynamin family members.
Finding and Recognizing the Right Partner during Meiosis.

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Sexual reproduction requires the unique cell division process known as meiosis, the goal of which is to produce haploid cells containing exactly one set of chromosomes. This requires that homologous chromosomes - pairs of chromosomes inherited from each parent - separate from each other after undergoing an intricate process of pairing, synapsis, and recombination. The primary questions we have investigated are what forces bring chromosomes together, and how each chromosome recognizes its true homologous partner among the chromosomes in the nucleus. We have explored these questions in the nematode C. elegans, which combines powerful molecular genetics with outstanding opportunities to directly image meiosis in both fixed and living animals. Our work has shown that special regions on each chromosome are required for both pairing and synapsis. These regions, known as "Homolog Recognition Regions" or "Pairing Centers," contain a high density of short, repetitive sequences, which recruit a family of zinc finger proteins known as HIM-8 and ZIM-1, -2, and -3. During early meiotic prophase, the binding of these proteins promotes interaction of chromosomes with the microtubule cytoskeleton, through a bridge of SUN and KASH domain proteins that spans the nuclear envelope. Disruption of microtubules sharply reduces chromosome dynamics and inhibits pairing between homologs. The cytoplasmic dynein motor complex is dispensable for pairing, but is required for chromosomes to "recognize" their partners and initiate formation of the synaptonemal complex, which stabilizes the relationship between homologs and enables them to undergo reciprocal recombination. Together these discoveries have shed light on longstanding mysteries about chromosome dynamics involved in reproduction.

Bioengineering (843 – 862)

Visualizing the Dynamics of Immunological Synapse Formation.

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Antigen-specific T lymphocyte activation, expansion, and acquisition of effector functions are critical components of an adaptive immune response. T-cells engage antigen presenting cell (APC) in a long lasting interaction that results in the formation of a structure known as the immunologic synapse (IS), a precursor to T-cell activation and proliferation. Here we describe a novel system to observe the modulation of protein organization and clustering which occurs at the is during the process of antigen recognition. at present, the most studied method for obtaining high-resolution images of the is in viable cells relies on the use of planar lipid bilayers [1], which can only provide a limited understanding of supramolecular clustering during is formation. Studies with these bilayers have revealed the is to consist of a central cluster of T-cell receptors (central supramolecular activation clusters [cSMAC]) surrounded by a ring of adhesion molecules (peripheral SMAC [pSMAC]). In parallel, large molecules like CD43 and CD45 are excluded from the is and migrate centrifugally to the distal supramolecular activation cluster (dSMAC) [2]. K562 cells modified to act as artificial APCs were used to study the formation of the is in T-cell populations. Cells were immobilized by micropit arrays which were functionalized to promote K562 cell adhesion. Human T-lymphocytes were purified from whole blood samples by negative selection and incubated with the sequestered K562 cells. Quantitative imaging of fluorescent TCR microclusters at the T-cell/APC interface has revealed assembly of microclusters at the pSMAC followed by transport towards the cSMAC. Simultaneously, LFA-1/ICAM-1 complex is assembled at the dSMAC, and transported to the pSMAC. This suggests that a modulation in the distribution

844/B2
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The cell microenvironment is composed of an extracellular matrix (ECM) with specific mechanoechemical properties that can direct cell behavior. Current hydrogels display a variety of these parameters, but they typically lack the ability to display these properties, such as adhesivity, in small, discrete "sticky" patches, such as the RGD binding site of fibronectin does in the context of an assembled matrix. Moreover, our ability to detect these regions by conventional light microscopy techniques is likely limited. Using a unique application of force spectroscopy on an atomic force microscope (AFM), we demonstrate here the ability to determine local changes in the 1) adhesive or 2) elastic properties of a material down to the resolution of the tip diameter, ~50 nm. Ligand-coated polyacrylamide (PA) hydrogels were interrogated with antibody-coated AFM tips to measure and map adhesion forces as a means of identifying these "sticky" patches. Fibronectin as a positive result is compared with albumin as a negative one. Micropatterned ligand study also verifies the specificity of this interaction. For elasticity, PA and polyvinyl pyrrolidinone (PVP) hydrogels were mapped, and while PA gels displayed a relatively uniform elasticity, PVP gels showed small regions where elasticity varied up to 50% from Eaverage. Domain area was found to be 0.2 - 0.6 μm2 in size. Though a sharp transition between 1 and 1.5% crosslinking was observed, indicating unique transition state in the PVP gel, it did not correlate with changes in surface roughness, pointing to a fundamental difference in the network structure of PVP versus PA gels. We feel that both applications demonstrate the resolution and versatility of this unique AFM application, which will enable detection of mechanical features well below the resolution limit of conventional light microscopy.

845/B3
Thermoplasmonic Nanoarrays to Manipulate Cell Adhesion.
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The cell, the most fascinating entity due to its complex and hierarchical micro-nanomachinery, uses specific transmembrane receptors, the integrins, to bind extracellular matrices (ECM) and to trigger subsequently its activity. Towards the development of lab-on-chip systems, control over these binding-sites would offer a unique way to organize, guide and release the entire cell or only part of it. Other than micropatterning and stimulus-responsive surface chemistries, only a limited number of technologies have been developed to culture cells in an effort to manipulate and investigate dynamic adhesive processes in more details. Our overall objective intends to develop a new generation of culture substrates engineered for cell manipulation with the abilities to interface specifically integrins and to generate heat nano-locally for the dissociation of the {integrin-ECM} bounds. The present paper describes our work on the design, characterization and effect of thermoplasmonic gold nanopatterned surfaces on cell adhesion. We find that gold nanoarrays, when made of 40 nm-particles functionalized with ECM-ligands (like the GRGDS peptide), are multifunctional because they support in-vitro cell growth, generate heat while excited by a green laser, and thus, change the state of adhered fibroblasts or melanoma from rounded to apoptotic. By simply adjusting the laser power, the released thermal energy can either be lethal for cells, or functional to release their focal adhesions from the array. When lethal (laser power >30 mW/cm2), the heat induces the fixation of the cells, which remain intact or detach within few
minutes, while a power of 20 mW/cm² causes the detachment of filopodes and the retraction of lamellipodia, which lead the cells to round up and to re-spread instantly after laser exposure. Our set up can easily reach the single cell resolution, offering a promising platform for microfluidic systems and new experiments in cell biology and nanophysics.

846/B4
Human Donor Variability and Dose-Dependant Effect of BMP4 on the Osteogenic Capacity of Human Umbilical Cord Stem Cells.
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Human umbilical cord stem cells (UCSCs) may be beneficial for use in cell therapy. Isolation of these cells from the umbilical cord results in a high harvest yield, and high expandability with stem cell phenotype stability. In this study, we examined human donor variability and dose-dependant effects of BMP4 on osteogenic capacity of UCSCs. Human UCSCs (n=8 donor populations) were stimulated with osteogenic media containing 0.1, 1, 10 and 100 ng/mL bone morphogenic protein 4 for 5-7 days and percent alkaline phosphatase (ALP) was examined. We subsequently prepared UCSC for the classic pellet assay (2.5E5 cells/pellet) for growth in control media, DMEM, or osteogenic media with 10ng/mL dose of (BMP4) for 10 and 28 days. Pellets were examined by micro-CT for matrix mineralization and were fixed in formalin for histological analysis. We observed that the most effective dose of BMP4 to increase ALP positivity varied with the donor population. In the pellet assay, we observed a larger amount of bone volume present in certain populations (UC80, UC82, UC84 and UC86). By day 28, we observed that these same populations did not continue to represent the most osteogenic cells, rather populations (UC79, UC81, UC83 and UC85) showed larger amount of bone volume present. To date, we have not detected any correlation between cell markers or ALP expression and mineralized matrix formation. Investigation shows that while all UCSC populations were capable of osteogenic differentiation, there was variability among populations. While previous studies showed remarkable phenotype consistency in the isolation of mesenchymal stem cells from the umbilical cord, UCSCs showed dose-dependant effects related to BMP4 concentration and length of stimulation. Findings have implications for individualized therapeutic approaches using mesenchymal stem cells. Whether autologous cells or allogeneic cells may be used in cell therapies, it will be important to examine the specific responsiveness of a given stem cell populations. Future studies are ongoing to examine the molecular differences among the populations with disparate osteogenic responses.

847/B5
Biophysical Cues Modulate Redox Potential in Cells.
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Objective: to determine if biophysical cues influence redox potential in cells involved with the drainage of aqueous humor from the eye. Methods: Human trabecular meshwork cells, TM-1, were plated onto hydrogels with Young’s moduli of 3, 30 and 100 kiloPascals (kPa) as well as on standard tissue culture plastic. The amount of reduced glutathione (GSH) in the TM-1 cells was measured using Ellman’s reagent. Cells were also plated onto fabricated polyurethane substrates that were either flat, or had a repeating pitch structures (equal sized grooves and ridges) of 400, 1400 or 4000 nm. After five days in culture, the cells were challenged with 0.5 mM H2O2 for two hours and then were harvested to measure GSH. Results: Cells grown on all compliant substrates had markedly elevated GSH. Preliminary data indicate cellular GSH was 5-30 fold higher on the hydrogels than on the plastic surfaces. The GSH was highest on the 30 kPa hydrogel and decreased somewhat as compliance decreased or increased around this value.
After the oxidative stress, cells grown on the 400 and 1400 nm pitch patterned substrates had significantly more GSH than those cells grown on the chemically identical flat surfaces or the cells on the 4000 nm pitch substrates. Conclusions: Our lab and others have previously shown that biophysical cues influence a wide menu of fundamental cell behaviors including orientation, cell proliferation, and gene expression. These data show that substrates that possess biophysical attributes (compliance and topography) that more closely approximates those observed In Vivo influence the redox potential of the cell. These results again demonstrate the importance of the biophysical environment on cell behaviors and composition. Cells In Vivo never perceive the hard flat surfaces that are typically used to culture them in vitro.

848/B6
Laminin Active Peptides Conjugated on Chitosan Membrane as a Cell Adhesive Matrix.
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Laminins are multifunctional heterotrimeric glycoproteins. at present, five α (α1-α5), three β (β1-β3), and three γ (γ1-γ3) chains have been identified and 16 isoforms can be formed by various combinations of each subunit. Laminins show tissue- and/or developmental stage-specific expression. Laminins-111, consisting of laminin α1, β1, and γ1 chain, plays a critical role during embryonic development and in maintaining the many biological functions of basement membrane. We previously identified about 50 active sequences from laminin-111 through the peptide screening method using over 673 peptides covering the entire sequence of laminin-111. Further, some biological active peptides from laminin-111 had been used to develop the artificial cell sheet by conjugated on chitosan membrane and found that cell-peptide-chitosan sheet accelerate the wound healing. Here, we conjugated 33 cell adhesive peptides from laminin-111 on chitosan membranes and assessed their biological functions to analyze the peptide specific functions. 24 cell adhesive peptide-conjugated chitosan membranes promoted human dermal fibroblasts (HDFs) attachment and 12 peptide-chitosan membranes showed cell spreading. HDF attachment to 9 peptide-chitosan membranes was inhibited by heparin and to 12 peptide-chitosan membranes was inhibited by EDTA. Additionally, HDF attachment to several peptide-chitosan membranes were inhibited by anti-β1 integrin antibodies. Further, 17 peptide-chitosan membranes promoted neurite outgrowth with PC12 cells. These results suggest that peptide-chitosan membranes interact with different cell surface receptors in a peptide dependent manner. These cell adhesive peptide-chitosan membranes could use to analyze the cellular functions mediated by specific cell surface receptors and have a potential to use as a biomedical material.

849/B7
Laminin B133 (Dsitkyfqmsle) Peptide Forms Amyloid-Like Fibrils and Interact with Integrin A2β1 and Syndecan.
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B133 peptide (DSITKYFQMSL, mouse laminin β1 chain 1298 - 1309) promotes cell attachment, neurite outgrowth, and amyloid-like fibril formation. Cell attachment to B133 is inhibited by both heparin and EDTA. We evaluated the essential residues for the B133 activities using various deletion peptides and sets of alanine substituted peptides. The N-terminus deletion peptide B133a (SITKYFQMSL) promoted cell attachment with well-organized actin stress fibers, although the amyloid-like fibril formation and neurite outgrowth activity were eliminated. Cell attachment to B133a was inhibited by EDTA and anti-integrin α2 and β1 antibody. The shortest peptide maintained the characteristics was B133d (KYFQMSL). In contrast, the C-terminus deletion peptide B133g (DSITKYFQMSL) showed cell attachment with membrane ruffling. Additionally, B133g promoted amyloid-like fibril formation and neurite outgrowth activity. B133g
promoted heparin dependent cell attachment and significantly stimulated attachment to syndecan over-expressed cells, suggesting that B133g promotes syndecan-mediated cell attachment. The Congo red analysis, which evaluated amyloid-like fibrils forming ability, indicated that amyloid-like fibril formation of B133g was stronger than that of B133. The shortest peptide maintained the activities was B133i (DSITKYFQM). Further, alanine substituted analyses of B133a and B133g suggest that the important residues for the integrin α2β1 mediated cell attachment are I, T, Y, F, M, and E, and for the syndecan mediated cell attachment are D and I. The N-terminus D residue was required for neurite outgrowth. Furthermore, amyloid-like fibril formation required D and I residues. These data suggest that the integrin α2β1 mediated cell attachment of B133 is critical for the B133i sequence, especially C-terminus E, and the syndecan-mediated cell attachment of B133 is required for the amyloid-like fibril formation and the N-terminal amino acid residues. In summary, the B133 peptide is bifunctional, and the integrin α2β1 and syndecan bindings are sequence and conformation dependent. These findings were useful to clarify the mechanism of amyloid-like fibril formation and create artificial materials with various biological functions.

850/B8
Hard Tissue Generation on Dental Implant Surfaces by 3-D Culture of Bone Marrow-Derived Cells with a Collagen Scaffold In Vitro.
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[Objective] We studied a host microenvironment, which regulated mouse osteoblasts to osseointegrate the implant by generation of mineralized matrix in a biocompatible and osteoconductive collagen scaffold onto the dental implant surfaces. [Materials and Methods] KUSA/A1 cells (JCRB1119; mouse osteoblastic cell line, bone marrow-derived mesenchymal cells) were used. The immature cells were seeded in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, penicillin (125 μg/ml)/streptomycin (200 μg/ml) in a humid 5% CO₂/air at 37°C. In order to induce differentiation in vitro, the KUSA/A1 cells were exposed to 50 μg/ml ascorbic acid (AA)+10mM β-GP (induction condition). Titanium alloy implants (Anodic Oxidized, HA-coated and 99% Ti) were used in the study. Collagen scaffold was used for 3-D culture of the mature KUSA/A1 osteoblasts. Step 1: the cells were monolayer-cultured in the DMEM+AA+β-GP (8.0×10⁴ cells/ml, 12-well). Step 2: the cell/collagen scaffold clusters were 3-D cultured in the DMEM+AA+β-GP (8.0×10⁴ cells/ml, 12-well). Step 3: subsequently, the cells embedded in the scaffold were cultured on the 3 types of implant surfaces (Anodic Oxidized, HA-coated and 99% Ti) (1×10⁵ cells/ml, 10cm dishes; DMEM+AA+β-GP). Light/phase-contrast microscopy, proliferation rate, ALPase activity, osteocalcin and calcium amount, and mineralization examinations for bone-like tissue formation were performed in attempts to study the histology and characteristics of the hard tissue generation. [Results & Conclusions] The KUSA/A1 osteoblasts, being 3-D cultured under induction condition in collagen type I scaffold having low antigenecity, actively proliferated and differentiated to deposit hard tissue in the scaffold in vitro. This study suggested that GTR concept tissue engineering of mesenchyme-derived cells obtained osseointegration on the implant surfaces in vitro, which would serve as a biocompatible and temporary 3-D framework followed by biodegradation and bone remodeling to optimize an implant-tissue interface -osseointegration- in vivo.

851/B9
Claudin as a Target Molecule for Mucosal Absorption of Peptide Drug.
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Objective: Recent progress in genomic and proteomic research provides us biologics (peptides, proteins and nucleic acids) as pharmaceuticals. Biologics is hydrophilic and poor in absorption. Paracellular pathway is an ideal route for absorption of biologics; however, epithelium functions as a barrier separating intra and outer body. Tight junctions (TJs) seal intercellular spaces in
epithelium and prevent free movement of solutes across epithelium through the paracellular route. Claudin, a four-transmembrane protein, comprises a protein family consisting of more than 20 members and is a key component in TJs-barrier. We previously found that a claudin-4 modulator, C-terminal fragment of Clostridium perfringens enterotoxin corresponding to 184-319 amino acids (C-CPE), enhanced jejunal absorption of dextran. In the present study, we investigated whether a claudin-4 modulator is a potent mucosal absorption-enhancer of a peptide drug.

**Methods:** Human parathyroid hormone derivative hPTH(1-34) was used as a peptide drug. Mucosal absorption was evaluated by rat jejunal, pulmonary and nasal administration of hPTH(1-34) and C-CPEs. Plasma hPTH(1-34) levels were measured by radioimmunoassay. Binding of C-CPEs to claudin-4 was assayed in enzyme-linked immunosorbent assay and Biacore assay. Modulation of TJ-barrier was evaluated using Caco-2 monolayer cell sheets. **Results:** C-CPE enhanced nasal absorption of hPTH(1-34) but did not jejunal and pulmonary absorption. C-CPE enhanced jejunal and pulmonary absorption when hPTH(1-34) was administered 4 h after C-CPE-treatment. C-CPE has poor solubility (0.3 mg/ml). Previous report indicated that N-terminal partly truncated C-CPE is a claudin binder with high solubility (10 mg/ml). We found that the truncated C-CPE bound to claudin-4, modulated TJ-barrier and enhanced jejunal absorption of dextran. The truncated C-CPE enhanced jejunal and nasal absorption of hPTH(1-34).

**Conclusion:** Claudin is a potent target molecule for development of mucosal absorption of peptide drug.

**852/B10**
Notch Ligand Endocytosis and Force Generation Measured by Optical Laser Tweezers.
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The Notch pathway is an evolutionary conserved signaling system used extensively throughout embryonic development and continues to function in the maintenance of tissues and stem cells in adults. Notch signaling requires a series of proteolytic cleavage events to release the Notch intracellular domain that functions directly in signal transduction. The Notch receptor is locked-down in a protease resistant state by a negative regulatory region (NRR) that protects an ADAM (A Disintegrin And Metalloprotease) cleavage site. Engagement with ligand-bearing cells is proposed to induce global conformational movements in Notch that unfold the NRR structure to expose the ADAM cleavage site and initiate proteolytic activation. Since endocytosis is critical for Notch signaling we are using optical tweezers to determine if ligand cells exert a pulling force on bound Notch and if this is dependent on ligand endocytosis. Specifically, we trap and place Notch-coated polystyrene beads next to cells expressing the Notch ligand Delta-like 1 (Dll1) and record the displacement of the Notch-bead as measured on a quadrant photodiode (QPD). Our preliminary findings identify a significant displacement of Notch-beads, indicating a force generated by the Dll1 cells on beads, and furthermore, the ability of our system to detect nanometer displacement. Importantly, Dll1 cells defective in endocytosis bind trapped Notch-beads but do not significantly displace them, suggesting that bead displacements by wild-type Dll1 are related to ligand endocytosis. To identify the specific endocytic pathway and characterize ligand cell endocytic forces measured with optical tweezers, we are using Dll1 endocytic mutants and RNA interference to deplete various endocytic proteins. Together our data provide support for the idea that endocytosis of ligand bound Notch exerts a force on Notch to facilitate proteolytic activation for downstream signaling.

**853/B11**
Drug-Loaded Fe3O4 Nanoparticles Induces Lung Cancer Cell Apoptosis through Caspase-8 Pathway Activation.
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Optimal formulation and route of delivery enhance cancer drug efficacy. In this study, a novel water dispersible oleic acid (OA)-triton X-100-coated Fe3O4 nanoparticles loaded with 10-Hydroxycamptothecin (HCPT) was developed to induce apoptosis in human lung cancer HCC827 cell line. HCPT partitions into the OA shell surrounding Fe3O4 nanoparticles and Triton X-100 that anchors at the OA-water interface confer the aqueous dispersion to the formulation. HCPT-loaded Fe3O4 nanoparticles were characterized by transmission electron microscopy (TEM), X-ray Diffraction (XRD), Fourier Transform Infrared (FT-IR) Spectroscopy, as well as High Performance Liquid Chromatography (HPLC) analysis. The HCPT-loaded Fe3O4 nanoparticles displayed excellent water dispersion, narrow size distribution, and were readily loaded with high doses of water-insoluble anti-cancer drug (HCPT). Our results demonstrated that HCPT-loaded Fe3O4 nanoparticles elicited an anti-proliferative effect in a dose-dependent manner in several cancer cell lines using MTT cell viability and flow cytometry assays. Apoptotic DNA ladder bands were clearly detected in human lung cancer HCC827 cells when treated with HCPT-loaded Fe3O4 nanoparticles. Furthermore, we found that compared with void Fe3O4 nanoparticles alone, HCPT-loaded Fe3O4 nanoparticles evoked synergistic effects by increasing cell apoptosis. Western blot analysis revealed an enhanced activation of caspase-8, 9, and 3 as well as an increased cleavage of poly (ADP-ribose) polymerase (PARP). Combined with annexin V-FITC apoptosis detection, our data suggests that HCPT-loaded Fe3O4 nanoparticles could be developed as a chemotherapeutic agent for increasing anti-cancer drug efficacy.

854/B12
Distinct Changes in Cell Proliferation within the Dentate Gyrus and Medulla Follow Spinal Cord Injury.
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Cell proliferation within the spinal cord is a well known concomitant of the inflammatory and degenerative events following spinal cord injury (SCI). Despite recent studies, much remains unknown regarding the role(s) and diversity of cell proliferation after injury, including whether it occurs in supraspinal regions distant from the lesion site. We sought to elucidate whether SCI elicits a proliferative response in the brain stem and/or forebrain, with the hypothesis that there is such a response within these regions. The rationale for this hypothesis is twofold: 1) axon degeneration, particularly anterograde degeneration, should stimulate glial cell proliferation associated with reorganization, and 2) many of the axons damaged by SCI are long motor pathways from brain stem nuclei and the cortex. To characterize the changes in cell proliferation within the medulla and dentate gyrus (DG) of adult, male C57BL/6J mice 96 hrs after SCI, we used a single injection of bromodeoxyuridine (BrdU) 30 minutes before sacrifice to label cells within S-phase. In the medulla, there is an 80% increase in the number of S-phase cells (p<0.05) predominantly in white matter tracts, suggesting that this response is glial. Double-labeling confirmed this to be a glial response, with GFAP+ and NG-2+ cells predominantly accounting for the S-phase cells (BrdU+). Moreover, this response is within regions that correlate with motor pathways damaged in the spinal cord. Interestingly, there is a 20 - 30% decrease (p<0.01) in the number of S-phase cells within the DG, a structure with no direct connections to the spinal cord. These data suggest that in response to SCI: 1) cell proliferation occurs in the brain, i.e., distant from a spinal cord lesion, 2) the increase in cell proliferation in the medulla is secondary to axonal degeneration, and 3) the decrease in cell proliferation within the DG is precipitated by a systemic signal elicited after SCI. Taken together, these results indicate that after SCI there are changes in cell proliferation within regions of the CNS distant from the site of SCI and these changes differ quantitatively and qualitatively in the areas studied.

855/B13
Furfural Prevents Growth and Induces Cellular Damage That Is Suppressed by the Overexpression of OYE2 in Saccharomyces Cerevisiae.
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A renewable fuel source is necessary to combat the rising economical and environmental concerns currently associated with fossil fuels. One possible renewable fuel is bio-ethanol from lignocellulosic biomass. However, there are many problems that need to be addressed before this alternative fuel can be realized. One major problem is the existence of multiple inhibitors found in a typical lignocellulosic hydrolysate. Some of the predominant inhibitors include furfural, hydroxymethylfurfural (HMF), and vanillin. These chemicals inhibit the growth and fermentation of the budding yeast, Saccharomyces cerevisiae. In order to fight the effects of fermentation inhibitors the stress protective gene, OYE2, was subcloned into the pRS425-MET25 expression vector. The enzyme encoded by OYE2 is a NADPH specific oxidoreductase linked to stress tolerance. To determine whether or not OYE2 overexpression increased furfural tolerance, we took exponentially growing yeast with either pRS425-MET25 or pRS425-MET25-OYE2 and exposed them to furfural. Aliquots of cells were removed at set time points to assay growth, reactive oxygen species (ROS) accumulation (dihydroethidium stain), membrane damage (mitochondrial targeted-GFP and FM 4-64), and chromatin damage (DAPI stain). Furfural induced the accumulation of ROS and cause cellular damage to membranes and nuclear chromatin. Yeast overexpressing OYE2 grew better in medium containing low and high amounts of furfural (25 mM and 50 mM, respectively). We predict overexpressing OYE2 will subsequently cause reduced damage to membranes and chromatin. These data demonstrate that overexpressing OYE2 provides protection to yeast against the industrial inhibitor furfural. This strategy indicates that genetically engineered yeast strains may prove valuable in improving the technology needed for lignocellulose to be converted into an efficient supply of bio-ethanol.

856/B14
Probing Cellular Mechanobiology in Three-Dimensional Culture with Collagen-Agarose Matrices.

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Cellular mechanobiology studies are currently limited by the lack of three-dimensional (3D) scaffolds that combine the biofunctionality of native extracellular matrix (ECM) proteins with the tunability of synthetic materials. Here, we introduce a novel biomaterial platform in which the biophysical properties of collagen I matrices are progressively altered by adding agarose. We find that agarose increases the bulk elasticity of 3D collagen ECMs over two orders of magnitude by forming a dense meshwork that intercalates between the entangled collagen fibers. Embedded glioma cells exhibit a pronounced transition to amoeboid motility accompanied by severe limitation of cellular invasion from multicellular spheroids as the agarose content of the hydrogels is increased from 0-1% w/v. Our results are consistent with a model in which agarose structurally couples and reinforces individual collagen fibers, simultaneously introducing steric barriers to cell motility while shifting ECM dissipation of cell-induced stresses from the non-affine deformation of individual collagen fibers to the bulk-affine deformation of a continuum network.

857/B15

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An adenoviral vector (Ad-GLP-1) was constructed that expresses bioactive glucagon-like peptide 1 (GLP-1) in the salivary glands of mice and its effectiveness at modulating glucose homeostasis was evaluated. The construct was engineered with the signal sequence of mouse growth hormone, to direct it into the secretory pathway, followed by a furin cleavage site and GLP-1(7-37) encoding an Ala to Gly substitution at position 8 to achieve resistance to degradation and
therefore increase its In Vivo half-life. When expressed in Neuro2A and COS7 cells, the active form of GLP-1 was detected specifically in the conditioned medium of the transduced cells by RIA; showed resistance to degradation by dipeptidyl-aminopeptidase IV; and induced the secretion of insulin from NIT1 pancreatic β-cells in vitro. In Vivo studies demonstrated that mice transduced with the Ad-GLP-1 in the submandibular gland had serum GLP-1 levels ~3-times higher than mice treated with the control Ad-Luciferase (Ad-Luc) vector. In fasted animals, glucose levels were similar between Ad-GLP-1 and Ad-Luc treated mice in keeping with GLP-1’s glucose dependent action. However, when challenged with glucose, Ad-GLP-1 treated mice cleared the glucose significantly faster than control mice. These studies demonstrate that the bioactive peptide hormone, GLP-1, normally secreted from endocrine cells in the gut through the regulated secretory pathway, can be engineered to be secreted into the circulatory system from exocrine cells of the salivary gland to affect glucose homeostasis.

858/B16
Kinesin-Calmodulin Fusion Protein as a Molecular Shuttle.
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Recently attention is focused on the application of molecular shuttles based on the kinesin and microtubule to the bionanotechnology such as nano-electro-mechanical systems (NEMS) or lab-on-a-chip. Previously biotin-avidin and antigen-antibody system have been used to attach target cargoes on kinesin. Although the systems are highly specific and tight, these are flawed as irreversible binding. In this study, we have developed molecular shuttle, which has reversible cargo loading system using calmodulin (CaM) and M13 peptide. We have designed kinesin K560 chimera protein fused with CaM at the C-terminal tail region of kinesin (K560-CaM). K560-CaM was expressed by E. coli. expression system and purified. The ATPase activity and the microtubules gliding activity of K560-CaM were almost in the normal range of the kinesin wild type. The Ca²⁺ dependent reversible binding of K560-CaM to the M13 peptide was monitored with HPLC using size-exclusion column (SEC-HPLC). Rotary-shadowing electron microscopy demonstrated that K560-CaM was observed as tetrameric configuration in the absence of Ca²⁺. on the other hand, in the presence of Ca²⁺, K560-CaM was observed as dimeric and tetrameric configuration. The Ca²⁺-dependent configurationally change of K560-CaM was also monitored by SEC-HPLC and analytical ultracentrifugation. Finally, we successfully observed that K560-CaM transports M13 peptide coupled with quantum dot along the rhodamine labeled microtubule in the presence of Ca²⁺ using TIRF-Microscopy. The transport velocity and run length of K560-CaM-Qdot are 305 ± 0.025 nm/s (mean± SE, N=48) and 5.19 ± 0.78 mm (mean± SE, N=48), respectively. on the other hand, in the absence of Ca²⁺, it is not observed K560-CaM-Qdot moving along rhodamine labeled microtubule.

859/B17
Flagellar Regeneration after Laser Severing.
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BACKGROUND: We study the control systems that determine eukaryotic flagellar length. Chlamydomonas reinhardtii cells construct two structurally identical flagella that are equivalent to mammalian cilia. By severing one of the two flagella, we perturb the system and measure the recovery kinetics in what Rosenbaum et al (1969) first described as the long-zero response. APPROACH: We use a tunable IR femtosecond laser to induce single deflagellation in microfluidically trapped cells. By time-lapse imaging, we track the length kinetics of the response. We then use the length kinetics to fit parameters of a simple 4-parameter ODE model of the system. METHODS: The microscope system is comprised of an inverted microscope, external
optics to direct the laser beams into the microscope, a CCD digital camera, and hardware-software for control of laser power, specimen stage and microscope stand focus and illumination. The ablation laser used in this system is femto-second Ti:Sapphire laser, which is tunable from 710 - 990 nm. Software for computer control of all hardware and image acquisition is custom coded in the Labview programming language. FINDINGS/CONCLUSIONS: Considerable cell-cell variation exists in the long-zero response. Some cells completely recover to initial flagellar lengths with the classical deceleratory kinetics described by Rosenbaum et al (1969). However, we also observe other distinct recovery responses, including recovery by deceleratory kinetics to a shorter flagellar length, the classic overshoot described by Rosenbaum et al (1969), complete resorption of flagella with no regrowth, partial resorption followed by recovery to a shorter length, and oscillating recovery where the flagella recover to the initial length but then shorten and later lengthen. From analyzing our results we can determine the parameter ranges in our ODE model that can account for the kinetics data. Overall, we note that cell-cell variation cannot account for all of the variation we see because some temporal variation exists within individual cells. In order to minimize such variation as a result of cell cycle or growth conditions, we conducted our experiments in light-dark synchronized cells as well as in gametes.

860/B18
Lipid Bodies in Chlamydomonas Reinhardtii.
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Given current interest in the development of technologies that harvest lipids from microalgae and convert them into diesel biofuel, we have initiated analysis of lipid-body (LB) biosynthesis in the unicellular green soil alga Chlamydomonas reinhardtii. When deprived of nitrogen after entering stationary phase in liquid culture, C. reinhardtii cells proceed to produce abundant cytoplasmic LBs, as well as abundant starch, via a pathway that entails a regulated autophagy program. After 48 hr of N-starvation, LB content in the wild-type strain has increased 15-fold. When starch biosynthesis is blocked in the sta6 mutant, LB content increases 30-fold, documenting that genetic manipulation can enhance algal LB production. Use of cell-wall-less strains permitted development of a rapid “popped-cell” microscopic assay to quantitate LB content per cell, and permitted gentle cell breakage and LB isolation. The highly purified LBs contain 90% triacylglycerol (TAG) and 10% free fatty acids (FFA). The fatty acids associated with the TAGs are roughly 50% saturated (C:16 and C:18) and 50% unsaturated, half of which is oleic acid (C:18:1). The FFA are roughly 50% C:16 and 50% C:18. The LB fraction also contains a minor amount of phospholipid, with the same phospholipid profile as whole-cell extracts, but lacks any chloroplast-specific galactolipids, attesting to the purity of the preparation; the phospholipids presumably form bilayers or monolayers around the LBs. Low levels of protein are also present; SDS-PAGE resolves 3 polypeptides migrating as 68K, 55K, and 27K species. Nitrogen-stress-induced LB production in C. reinhardtii has the hallmarks of a discrete pathway that should be amenable to genetic manipulation and additional enhancement.

861/B19
Overexpression of ZWF1 and YDR049W Protect Yeast from Oxidative Damage Caused by the Fermentation Inhibitor Furfural.
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Currently our nation is facing many environmental and economical concerns, raising the need for an alternative and renewable fuel source. One such fuel source that has been proposed and tested is bio-ethanol. Unfortunately before an ethanol-based fuel industry can be achieved, several complications must be addressed. While the current process of producing bio-ethanol using Saccharomyces cerevisiae (baker’s yeast) using corn starch is quite efficient, our nation lacks the amount of corn needed to produce the desired amount of bio-ethanol for fuel. However, alternative substrates that would replace cornstarch substrates, such as lignocellulosic biomass waste, produce multiple inhibitors during the fermentation process. These inhibitors prevents
yeast from efficiently producing ethanol. These inhibitors include furfural, hydroxymethylfurfural, and vanillin. In order to overcome this problem, robust yeast strains are needed that can survive the stresses present during the industrial fermentation process. Our objective is to genetically engineer a more robust yeast strain using genes we have previously identified as functioning in furfural tolerance. Two of these genes are ZWF1 and YDR049W which were both subcloned into the pRS425-MET25 expression vector. These subclones were used to see if their overexpression would increase growth and decrease cellular damage to membranes, actin cables, and chromatin as assayed using fluorescence markers in the presence of furfural. When ZWF1, a pentose phosphate pathway gene, was overexpressed yeast grew better in the presence of furfural and furfural induced damage to membranes, actin cables, and chromatin was less severe. The overexpression of YDR049W was less effective at reducing furfural sensitivity compared to ZWF1 overexpression. Overexpressing ZWF1 and possibly other genes will lead to more robust yeast that are capable of surviving the stresses associated with the industrial fermentation of lignocellulosic biomass waste.

862/B20
Engineering of Fatty Acid Production and Secretion in Saccharomyces cerevisiae. G. Ribeiro, M. Côrte-Real, B. Johansson; Departamento de Biologia, Universidade do Minho, Braga, Portugal

Production of renewable liquid biofuels that can substitute fossil fuel, has emerged as a major challenge for applied biology. Biodiesel, in the form of fatty acid esters, produced by oleaginous microorganisms could be an attractive alternative, since the utilization of diesel fuel is more efficient than for example ethanol. Oleaginous yeasts may accumulate very high (60%) levels of intracellular lipids but two drawbacks are the relatively complicated extraction process and the subsequent transesterification with the accompanying glycerol by-product formation. The objective of this work is to apply metabolic engineering of fatty acid synthesis and secretion in the model yeast S. cerevisiae in order to create a microorganism able to produce and secrete free fatty acids or fatty acid esters. S. cerevisiae is a proper model, since lipid metabolism has been studied extensively and all genes encoding enzymes directly involved in lipid synthesis are known. This organism has also been reported to acquire oleaginous properties by no more than three genetic modifications(1). In the yeast S. cerevisiae, activation of exogenous long-chain fatty acids to coenzyme a derivatives, prior to metabolic utilization, is mediated by the fatty acyl-CoA synthetases Faa1p and Faa4p. It has been shown that free fatty acids are secreted from a FAA1,4 double mutant(2). This modification will be combined with modifications of core fatty acid elongation, such as overexpression of acetyl-CoA synthetase (Acs1p), in an attempt to improve fatty acid production rate. Essential for this work is to facilitate a biological platform for efficient fatty acid or lipid production. In this work, the “delitto perfetto” method (3) was applied to delete these two fatty acyl-CoA synthetases generating genetically clean strains without markers or bacterial DNA. Results show that this technique can be used to generate multiple knockouts by recycling the marker gene. 1.Kamisaka Y, Tomita N, Kimura K, Kainou K, Uemura H. Biochem J. 2007 Nov 15;408(Pt 1):61-68. 2.Michinaka Y, Shimauchi T, Aki T, Nakajima T, Kawamoto S, Shigeta S, et al. J of Biosci and Bioeng. 2003 ;95(5):435-440. 3.Storici F, Resnick MA. Methods Enzymol. 2006 ;409:329-45.

Cancer II (863 – 896)

863/B21
Beta-Arrestin1 as the Pivotal of Multiple Signals Promotes Leukemia Progression. H. Liu, J. Long, P. Zhang, Z. Tu, L. Zou; ^1Center for Clinical Molecular Medicine, Children's Hospital, Chongqing Medical University, Chongqing, China, ^2Department of Clinical Biochemistry in Laboratory Faculty, and the Key Laboratory of Laboratory Medical Diagnostics in the Ministry of Education, Chongqing Medical University, Chongqing, China
Arrestins (Arrs) are scaffold proteins consisting of four members: β-arrestin1 (β-Arr1), β-arrestin2 (β-Arr2), α-arrestin, and s-arrestin. Only β-Arr1 and β-Arr2 are ubiquitously expressed, and mediate desensitization, sequestration, and recycling of G protein-coupled receptors (GPCRs). Recent studies showed that β-arrestins also serve as modulators of intracellular signals, including ERK, JNK3, ASK1, IGF-1 and PI3K-Akt, through which regulate various cellular functions in both normal and malignant cells. β-Arrs are found to be associated with tumor signaling pathways such as TGF-β1, P53/MDM2 and NF-κB. Our previous data have disclosed that β-Arr1 promotes MMP9 activity and tumor angiogenesis through PI3K signals by providing a suitable microenvironment for tumor progression. However, little is known about β-Arrs function in leukemia. Here we collected the bone marrow (BM) and periphery blood (PB) samples from 95 patients with newly diagnosed leukemia, 36 volunteers without malignant hematological diseases as the control, to investigate the expression of β-Arrs. The results showed that all the mRNA and protein levels of β-Arr1 and β-Arr2 increased significantly in both BM and PB samples of leukemia patients (P<0.05 or P<0.01) comparing with controls. However, there was no significant difference of β-Arr expressions among the diverse subtypes of leukemia (AML, ALL and CML) samples (P>0.05), but the relative expression of β-Arr1 was always higher than β-Arr2 in the same patient. We further found that over-expression of β-Arr1 could promote leukemia cells to proliferate and vice versa. Interestingly, PI3K/Akt, JNK/STAT, NF-κB and Wnt/β-catenin pathways were all activated in the leukemia cells, and could be reduced by knocking down β-Arr1. In addition, the expressions of downstream genes of these signals including cyclin-D1, c-fos and c-myc were increased too. Furthermore, the expressions of cyclin-D1, c-fos and c-myc could be suppressed by blocking β-Arr1. Our data firstly reveal a pivotal role for β-arrestin1 in leukemia cells, in which β-Arr1 plays the hinge of multiple signals by promoting cell proliferation for the pathogenesis and progression of leukemia.

864/B22
A Novel Mechanism for Chemo-Immune Modulation: Cancer Chemotherapy-Induced Elevation of MHC I and ISG15 Expression.
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It has long been recognized that some chemotherapeutic agents, including cyclophosphamide, gemcitabine, cisplatin, and 5-flourouracil, have immuno-modulating functions, and can be used in combination with immunotherapy for better tumor killing efficacy. A number of mechanisms have been proposed including the suppression of regulatory T cells. However, the underlying mechanism for the chemo-immune modulation remains largely unclear. In this study, we showed that topotecan (TPT), as well as other chemotherapeutic agents (etoposide, taxol, vinblastine, and cisplatin), can upregulate the expression of Major Histocompatibility Complex Class I (MHC I) and Interferon-Stimulated Gene 15 (ISG15) in tumor cells, through induction of interferon-beta (IFN-b). Elevated MHC I on tumor cell surface sensitizes the cell to antigen-specific cytotoxic T lymphocyte killing. ISG15, an interferon-inducible cytokine, is known to stimulate T cell and induce natural killer cell proliferation. Here, we showed that conditioned media from TPT-treated ZR-75-1 cells induced elevation of MHC I and ISG15 expression in untreated cells, indicating the secretion of a transmissible factor(s) that is (are) responsible for the induction. Furthermore, we demonstrated that IFN-b was responsible for the TPT effect on MHC I and ISG15, as interferon receptor 1 subunit (IFNAR1) siRNA knockdown diminished the induction, and neutralizing antibody against IFN-b, but not IFN-a, blocked the induction. Finally, we found that NF-kB activation inhibitors block the MHC I and ISG15 induction by TPT, suggesting a pivotal role of NF-kB in mediating the TPT effect on MHC I and ISG15. In conclusion, we propose that induction of MHC I upregulation by chemotherapeutic agents such as TPT may be responsible for at least in part the immuno-modulatory effect of cancer chemotherapy. This chemo-immune modulation is likely to be through a series of events involving chemo-induced NF-kB activation, IFN-b secretion, and elevated expression of MHC I and ISG15.
865/B23
Water Soluble Wolfberry Phytochemicals Induce Cell Cycle Arrest and Apoptosis in Cancer Cells.
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Objective: The objective of the current study is to determine the effect of water soluble phytochemicals of Lycium barbarum (wolfberry), a common Chinese herbal medicine, on cell viability and induction of apoptosis in cancer cell lines. Methods: Three cancer cell lines, including human colon cancer SW480, human leukemia Jurkat, and feline lymphoma F1B cells, were used in this study. Wolfberry fruits were purchased from the local grocery store. The water soluble wolfberry extracts (i.e., wolfberry phytochemicals) were isolated, freeze dried, and re-dissolved in cell culture media. Total polyphenols were analyzed by Folin-Ciocalteu method. Antioxidant activity was determined by ferric-reducing antioxidant power (FRAP) assay. Cell cycle arrest was determined by flow cytometry. Western blotting was used to monitor alteration of protein expression. Results: Total polyphenols in water soluble wolfberry extracts was 1873 mg gallic acid / 100 g dry weight, and antioxidant activity of the extracts was 7771.2 micromol Trolox/ 100 g dry weight. Water soluble wolfberry extracts inhibited growth of SW480, Jurkat, and F1B cells in a dose dependent manner. The growth inhibition effects were found to be 1mg/ml at 48 hours after treatments in SW480 and Jurkat cells. Flow cytometry results indicated that the treatments of wolfberry extracts induced cell cycle arrest in G2M phase and consequent apoptosis at 48 hours after treatments. Protein levels of cyclin B1, cleaved caspase-3, and cleaved poly(ADP-ribose) polymerase (PARP) were significantly increased by the treatments as determined by Western blotting. Conclusions: Water soluble wolfberry phytochemicals were effective in inhibiting cell growth and inducing apoptosis in cancer cells in culture. Further studies on identification of major active components and potential synergy of the wolfberry phytochemicals may help to develop novel complementary therapeutic agents and/or dietary regimens for cancer prevention and treatment.

866/B24
Controlling Tumor Inflammation: Role of Integrin \(\alpha_4\beta_1\) in M1-M2 Macrophage Polarization.
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Two types of macrophages linked to cancer, M1 and M2, secrete cytokines and immune response factors that inhibit or stimulate tumor progression, respectively. By increasing the M1 to M2 ratio in the tumor microenvironment, the fate of the tumor can be controlled. Previous research has shown that the accumulation of macrophages in tumors is regulated by the integrin \(\alpha_4\beta_1\). Blocking the function of this integrin can reduce tumor growth, inflammation, and metastasis. Therefore, we investigated the role of \(\alpha_4\beta_1\) in controlling the M1/M2 macrophage ratio. Our experiment used a mouse model with subcutaneous injection of Lewis Lung Carcinoma (LLC) tumor cells in wild type (WT) and mutant mice (Y991A or YA), which express a non-functional \(\alpha_4\) subunit in the cytoplasmic region of the tail. First, we isolated tumor infiltrating macrophages using magnetic-activated cell sorting (MACS). Then, we performed quantitative PCR for different cytokines to distinguish M1 and M2 macrophages. We observed a downregulation of immunosuppressive M2 cytokines (TGF-\(\beta_1\), but non-significant reduction in IL-10. However, there was an upregulation of immunostimulatory M1 cytokines (IL-12, IFN-\(\gamma\), TNF-\(\alpha\)) in the Y991A mice. This may indicate a general reduction in tumor inflammation, angiogenesis, lymphangiogenesis, and metastasis, as well as increased anti-tumor immunity. The ability to manipulate the M1/M2 ratio may lead to better prognosis and novel treatments for future cancer therapy.

867/B25
Different Induction of Granulocytic Differentiation by HDAC Inhibitors and All-Trans Retinoic Acid in Human Acute Promyelocytic Leukemia HL-60 and NB4 Cells Is Associated with the Involvement of C/EBP\(\alpha\), PU.1 and G-CSF Receptor Signals.
CCAAT/enhancer-binding protein-alpha (C/EBPα) and PU.1 are the basic transcription factors that control differentiation-related genes, including G-CSFR and human neutrophil elastase (HNE). Here, we analyzed a role of C/EBPα and PU.1 in the human acute leukemia HL-60 and NB4 cells in association with a modified chromatin structure by histone deacetylase (HDAC) inhibitors. We demonstrated that diverse HDAC inhibitors - FK228, sodium phenyl butyrate and vitamin B3 in combinations with all trans-retinoic acid (RA) effectively accelerated and enhanced HL-60 but not NB4 cell granulocytic differentiation detected by NBT test and CD11b or CD34 expression by flow cytometric analysis. HDAC inhibitors induced a time- and dose-dependent accumulation of hyper-acetylated histone H4 in both cell lines with the delay in NB4 cells. Chromatin immunoprecipitation analysis revealed that 6 h-pretreatment with sodium phenyl butyrate before the combined treatment with RA and vitamin B3 caused histone H4 acetylation in the G-CSF and the G-CSF receptor promoter regions in HL-60 cells. The time-dependent different induction of HL-60 and NB4 cell differentiation was coincident with different activation of C/EBPα and PU.1 binding activity to the G-CSFR and the HNE promoters in electrophoretic mobility shift assay. The results indicate that epigenetic events, such as histone acetylation, might be involved in the activity modulation of the key transcription factors responsible for the induction of leukemia cell granulocytic differentiation.

868/B26
B-Catenin Mediates FGF-7-Induced Ocular Surface Squamous Neoplasia in the Transgenic Mice.
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We have recently reported that excess fibroblast growth factor-7 (FGF-7) in Krt12rtTA/rtTA/tetO-FGF-7 double transgenic mice induced by doxycycline (Dox) resulted in a corneal epithelial tumor resembling human ocular surface squamous neoplasia (OSSN). In this study, we further investigate the role of β-catenin in mediating FGF-7 induced corneal tumorigenesis. We examined the role of FGF-7/β-catenin signaling in OSSN. Pannus surgically removed from patients exhibited excess FGF-7 and nuclear β-catenin. In vitro, FGF-7 induces stabilization and nuclear translocation of β-catenin in human corneal epithelial cells. Conditional Ctnnb1 loss- and gain-of-function mutants were expressed in tet-On Krt12rtTA/rtTA/tetO-FGF-7 mice that manifested OSSN upon Dox induction. Loss of Ctnnb1 from the corneal epithelium did not have significant effects on corneal homeostasis, but abolished OSSN caused by FGF-7 in Dox-treated Krt12rtTA/rtTA/tetO-FGF-7/tetO-Cre/Ctnnb1lox(E2-6)/lox(E2-6) mice. In contrast, in Dox-treated Krt12rtTA/rtTA/tetO-Cre/Ctnnb1floxed E3/Wt mice, expression of constitutive active β-catenin in differentiated (Keratin 12-positive) corneal epithelial cells trigger de-differentiation of corneal epithelial cells into more primitive progenitor cell phenotype expressing p63, and suppression of K12 keratin and Pax-6. The results suggest that β-catenin plays a pivotal role in mediating the formation of OSSN. Thus, the administration of inhibitor(s) that prevents activation of β-catenin may be beneficial for treating OSSN. β-catenin in human corneal epithelial cells.

869/B27
A Critical Concentration of CD8+ T Cells Is Required to Block Growth of B16 Murine Melanoma Cells.
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Li et al (PNAS 99:8289-94, 2002) derived an equation that can be used to calculate the critical neutrophil concentration, the concentration of neutrophils required to hold bacterial growth constant. We report here that this equation can be used to calculate the critical concentration of CD8+ OT-1 cells (CTC) required to control growth of SIINFEKL peptide-pulsed B16 melanoma cells and of SIINFEKL-B16 cells in spheroids in three-dimensional collagen-fibrin gels. Combined with clonogenic assays for B16 cells, the collagen-fibrin gel system employed in these studies are 3,500 to 4,000-fold more sensitive than the packed-cell-pellet type assays generally used to measure CD8+T-cell cytolytic activity. We report that the CTC is 3.9 x 10^7 OT-1 cells/ml collagen-fibrin gel, and that ≥10^7 OT-1 cells/ml gel kill 100% of 2 x 10^7/ml SIINFEKL-B16 cells in these gels in 7 days. The efficiency of T-cell killing (as measured by the killing constant, k, for OT-1 cell-mediated cytolysis of SIINFEKL-B16 cells [~8 x 10^-10 ml/OT-1 cell/min]) is comparable to that calculated for LCMV-specific CD8+T-cell-mediated cytolysis of LCMV-peptide-pulsed splenocytes in mouse spleen in vivo, and ~7 fold greater than for OT-1 cell killing of ova peptide-expressing B16 cells in B16 melanomas in vivo. Accordingly, the CTC for OT-1 cell killing of ova-B16 cells in ova-B16 melanomas In Vitro is ~9 fold lower in collagen-fibrin gels than OT-1 killing of ova peptide-expressing B16 cells in B16 melanomas in vivo. Thus collagen-fibrin gels provide a useful system with which to explore kinetic and functional relationships between CD8+T-cell effector functions In Vitro and in vivo. Supported by NIAID grant AI20516.

870/B28
A Role for CtIP in DNA Interstrand Cross-Link Repair.
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A DNA interstrand cross-link (ICL) is a highly toxic form of DNA damage that consists of a covalent bridge between complementary strands of the DNA double helix. An ICL provides a physical barrier that prevents DNA strand separation during replication and transcription, thus causing cell death if not properly repaired. In order to characterize which factors are required for initiating the repair of ICLs in human cells during S-phase, we employed a novel laser scissor assay that allows us to generate ICLs in specific regions of the cell nucleus by activating psoralen by irradiation with laser light at the effective wavelength of 365 nm. We found that CtIP, a conserved protein that acts cooperatively with the Mre11-Rad50-Nbs1 (MRN) complex in double strand break repair, is required for ICL repair. We found that enrichment of the repair proteins γH2AX, ATM, FANCD2 and Rad51 at nuclear regions containing ICLs is reduced in CtIP siRNA depleted cells compared to control siRNA treated cells. In contrast, depletion of the nucleases Mus81 or Xpf, which have been proposed to act early in ICL repair, had no effect on γH2AX levels at laser activated ICLs. We propose that CtIP acts in concert with MRN to initiate repair at replication forks stalled at ICLs enabling recombination mediated repair to proceed.

871/B29
Independent, Stochastic Competition between Mitotic Slippage and Death Activation Governs Live-Or-Die Decisions in Response to Kinesin-5 Inhibitors.
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After a prolonged treatment of Kinesin-5 inhibitors (K5Is), a class of anti-mitotic drugs now in phase I clinical trial, cancer cells display large intra- and interline variation in their live-or-die decisions. It has been proposed that cell fates are dictated by two competing pathways, one involving death activation, the other protecting cyclin B1 degradation/mitotic slippage (Gascoigne and Taylor, 2008). This model has not been rigorously tested both experimentally and computationally: for example, it is unclear whether these two pathways are mechanistically coupled, or independent. In this study, we present evidence supporting that they are independent pathways, and the stochastic, kinetic competition between the two determines cell fates. We
developed methods to separate the two pathways experimentally in four human cancer cell lines with different degrees of death sensitivity. This allowed us to measure kinetics that are normally hidden in drug treatment alone, i.e. cyclin B1 degradation kinetics in death-sensitive cell lines, and death kinetics during mitotic arrest in death-resistant lines that tend to slip out of mitosis before they die. We then used Monte-Carlo methods to randomly select events from these two experimentally measured probability distributions, and showed that for all four lines, the simulated behavior matched the observed behavior when both processes occur together in K5I treatment. We further showed experimentally, that cyclin B1 degradation kinetics is unaffected when death kinetics is stimulated by co-treatment with TRAIL, and death kinetics is unaffected when cyclin B1 degradation is prevented by Cdc20 knockdown. This again argues the two pathways are independent. Taken together, our results reveal that intra- and interline variation of cell fate in response to K5I originates from independent, stochastic competition between death activation and cyclin B1 degradation.

872/B30 ABSTRACT WITHDRAWN

873/B31
Expression of βig-H3 in Cancer Stroma Derived from Mesenchymal Stem Cells in the Lung Adenocarcinoma.
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βig-h3 plays a pivotal role in tumorigenesis and metastasis as an extracellular adhesion molecule. However, the molecular mechanisms involved in the expression of periostin in tumor tissues are largely elusive. Lysophosphatidic acid (LPA) is a bioactive lipid that promotes cancer cell adhesion and motility through activation of G protein-coupled receptors. In the present study, we demonstrate for the first time that LPA stimulates the expression of βig-h3 in human adipose tissue-derived mesenchymal stem cells (hASCs). The LPA-induced expression of βig-h3 was abrogated by pretreatment of the cell with Ki16425, an antagonist of LPA receptors, or by depletion of LPA receptor 1 expression with small interfering RNA (siRNA) or small hairpin RNA (shRNA). Furthermore, conditioned media from A549 human lung adenocarcinoma cells induced expression of βig-h3 and the expression of big-h3 was completely abrogated by treatment with Ki16425 or silencing of LPA receptor 1, suggesting that LPA is involved in the cancer conditioned media-induced βig-h3 expression. In addition, treatment of A549 cells with recombinant βig-h3 protein enhanced proliferation, adhesion, and invasion of A549 in vitro. Taken together, these results suggest that cancer-derived LPA stimulates secretion of βig-h3 from hASCs and βig-h3 play a key role in tumorigenesis as a paracrine factor.

874/B32
Differential Effects of Hypoxia and Actin-Sequestering Protein, Thymosin Beta-4 on HeLa Cell Survival and Death through ROS-Mediated HIF-1α Stabilization.
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Here, we investigated the effect of actin-sequestering protein, thymosin beta-4 (TB4)-induced hypoxia on tumor cell survival and death. The increased TB4 level was detected in human gastric and colon cancer mass. Hypoxia inducible transcription factor (HIF)-1α was stabilized by TB4 in HeLa cervical tumor cells. When cells were treated with TB4 protein (TB4P), a significant increase in intracellular ROS level was detected. The increased level of HIF-1α by TB4P was reduced by the treatment with N-acetylcysteine (NAC), a well-known ROS scavenger. A basal level of tumor cell death was reduced by TB4. In contrast, hypoxia increased ROS and the basal level of tumor cell death. While HIF-1α stabilization was independent of the relative intracellular ROS level, the effect of exogenous H2O2 on cell density was different. While 10nM H2O2 increased cell density, more than 10mM H2O2 reduced it. NAC treatment attenuated TB4-increased cell density. Paclitaxel-induced tumor cell death was reduced under all hypoxia conditions but no decrease by TB4 was detected when cells were transfected with HIF-1α-siRNA. Paclitaxel-induced B16F10
mouse melanoma regression was physiologically inhibited in TB4-transgenic mice compared to wildtype mice. Taken together, these findings demonstrate that TB4 could play a role in tumor cell resistance to anticancer agents through ROS-mediated HIF-1α stabilization. Our data also show that the effect of TB4-induced hypoxia on enhancing cell survival is different from the effects of oxygen-deficient hypoxia on increasing cell death. These results suggest that TB4 could be a novel modulator to control tumor cell survival and death through the regulation of ROS production.

875/B33
Photodynamic Therapy in an HCT116 Human Colon Cancer Xenograft Model Using a Chlorine-Based Photosensitizer DH-II-24.
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While photodynamic therapy (PDT) has been recognized as a promising therapeutic modality for the treatment of various cancers and diseases, developments of effective photosensitizers are highly desired to improve the prospect for the use of PDT. In this study, we evaluated DH-II-24, a new photosensitizer, for antitumor PDT In Vitro and in vivo. Loaded into human colorectal carcinoma cells (HCT116), DH-II-24 was primarily accumulated in mitochondria, lysosomes and endoplasmic reticula. Administration of DH-II-24 followed by light exposure induced necrotic cell death in a dose-dependent manner, whereas DH-II-24 in the absence of light displayed a minimal cell death. In order to investigate the distribution and pharmacokinetics of the photosensitizer in vivo, DH-II-24 was intravenously injected to female BALB/c nude mice. Fluorescence imaging In Vivo showed that DH-II-24 was rapidly distributed across the entire body and then mostly eliminated at 24 h. Next, effectiveness of DH-II-24-mediated PDT was examined on colorectal carcinoma xenografts established subcutaneously in BALB/c nude mice. DH-II-24 (1 mg/kg, i.v. administration) followed by light exposure significantly suppressed growth of xenograft tumors, compared to light exposure or DH-II-24 alone. Histological examination revealed necrotic damage in PDT-treated tumors, concomitantly with severe damage of tumor vasculature. These results suggest that DH-II-24 is a potential photosensitizer of photodynamic therapy for cancer.

876/B34
Functional Analysis of Glucocorticoid-Regulated Genes in Childhood Acute Lymphoblastic Leukemia.
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Glucocorticoids (GC) have pronounced effects on metabolism, differentiation, proliferation and cell survival. In certain lymphocytes and lymphocyte-related malignancies, GCs inhibit proliferation and induce apoptosis. This has led to their therapeutic use in malignant lymphoproliferative disorders. Our lab addressed the molecular basis of the anti-leukemic GC effects by comparative gene expression profiling using acute lymphoblastic leukemia (ALL)-children and related experimental systems. This provided a list of candidate genes for the anti-leukemic GC effects. Two interesting members of this list are SIK1 and PFKFB2 that have both been implicated in energy metabolism and survival. The former exerts pro- and anti-apoptotic functions in various biological systems, the latter is a regulator of glucose metabolism, a phenomenon that plays a crucial role in lymphoid cell death decisions. A third candidate, ZBTB16 is a transcriptional repressor involved in differentiation of myeloid cells and translocated to the RAR-alpha receptor in acute promyelocytic leukemia. To test their possible involvement in GC-induced cell death and/or cell-cycle arrest, we generated lentiviral constructs for conditional expression of SIK1, ZBTB16 and the 2 major PFKFB2 splice variants. Using these constructs, we established several stable transfected subclones of the human ALL cell line CCRF-CEM with tetracycline-regulated expression of our genes. Functional analyses showed a different role of the two isoforms of PFKFB2 in modulating GC-induced cell death. While PFKFB2-15A was without
effect, overexpression of PFKFB2-15B entailed accelerated GC-induced apoptosis suggesting that deregulation of glucose metabolism by GC may contribute to GC-induced cell death in ALL cells. on the other hand, overexpression of ZBTB16 resulted in pro-survival effects, explained by its inhibitory role on GC-dependent transcription. Overexpression of SIK1 in CCRF-CEM subclones had no detectable effect on survival or cell cycle progression, neither alone nor in combination with GC. It may, however, contribute to other effects of GC on innate and adapted immunity. To address this further, we are currently investigating the role of this gene in SIK1-KO mice.

**877/B35**

**Elevated IFIT2 Expression Promotes Oral Squamous Cell Carcinoma Differentiation.**  
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Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2) is one of most responsive interferon-stimulated genes to interferons, viruses, and a variety of agents. However, the biological functions of IFIT2 are poorly understood. Our previous studies have shown that elevated IFIT2 protein expression was positively associated with tumor differentiation status and better prognosis in patient with oral squamous cell carcinoma (OSCC). However, whether IFIT2 plays any role in OSCC differentiation is currently unknown. To this end, confluence-induced differentiation model was established and several well-known differentiation marker proteins, such as Cytokeratin10/Cytokeratin 13 (CK10/13) and involucrin were used to define distinct stages of OSCC differentiation. We found that IFIT2 expression was increased in early differentiation and sustained higher in terminal differentiation. We observed that IFIT2 expression was higher in superficial cell layer as compared with basal cell layer by using immunostaining assay. These results confirmed that IFIT2 expression was positive associated with OSCC differentiation status. Furthermore, we overexpressed IFIT2 protein in OSCC cells, and select two stable clones to study whether IFIT2 could regulate OSCC differentiation. Under pre-confluence condition, CK10/13 and involucrin levels were increased by 12.8 and 1.8-fold, respectively, in IFIT2-overexpressed cells as compared with cells transfected with control plasmid. Similarly, involucrin level was increased by 5.8-fold in IFIT2-overexpressed cells as compared with cells transfected with control plasmid under post-confluence condition. These results indicate that elevated IFIT2 expression may regulate OSCC differentiation. Moreover, Immunoprecipitation-western and confocal image analysis showed that IFIT2 interacted with CK10/13. Thus, elevated IFIT2 expression may be a pre-requisite of OSCC differentiation through binding of CK10/13.

**878/B36**

**STIM1 Remodels Calcium Signaling for Cancer Cell Proliferation and Metastasis.**  
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Store-operated calcium entry (SOCE) is the predominant Ca2+ entry mechanism in nonexcitable cells. Recent studies have identified two genes, STIM1 (stromal interaction molecule 1) and Orai1 (also named CRACM1), that are responsible for SOCE activation. Here we demonstrate the important role of STIM1 in promoting cancer cell proliferation and metastasis. Overexpression of STIM1 enhanced the invasive migration of cervical cancer cells, whereas knockdown of endogenous STIM1 significantly inhibited that of cervical cancer cells. The molecular mechanisms by which STIM1 regulated the invasive migration involved the modulation of focal adhesion turnover and subsequently cellular contraction force. Knockdown of STIM1 inhibited cervical cancer cell proliferation and caused cell cycle arrest at S and G2/M phase by p21 upregulation and Cdc25C downregulation. In severe combined immune deficiency mice model, we subcutaneously injected the clones of cervical cancer cells with differential STIM1 expression. STIM1 overexpression enhanced tumor formation and increased tumor vessel density. In contrast, STIM1 knockdown retarded tumor growth and decreased vessel density. Treatment with
SOC channel inhibitors, SKF96365 and 2-aminoethoxydiphenyl borate (2-APB), also inhibited tumor formation and angiogenesis. In 75% of surgical specimens we examined (n=30), STIM1 was overexpressed in cervical cancer tissues. In addition, the expression level of STIM1 in tumor tissues was closely correlated with tumor size and pelvic lymph node metastasis, which are two major poor prognostic factors for early-stage cervical cancer. Thus, the processes of proliferation and metastasis remodeled by STIM1 overexpression may provide the therapeutic intervention.

879/B37
Inhibitory Effects of miR-200a on Nasopharyngeal Carcinoma Cell Growth, Migration and Invasion Are Mediated by Different Targets.
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Abstract: Nasopharyngeal carcinoma (NPC) is a cancer originating in the nasopharynx with viral, dietary, and genetic factors implicated in its causation. The molecular basis of NPC pathogenesis has not been well defined. Recently studies have shown that miR-200a was downregulated in NPC by microRNA microarray analysis. To further investigate the function of miR-200a in NPC carcinogenesis, we analysed miR-200a expression in a panel of different differentiated NPC cell lines, including C666-1 (un-differentiation), CNE-1 (high differentiation), CNE-2 and HNE1 (low differentiation). We found that miR-200a expression was relatively lower in un-differentiated C666-1 than in highly differentiated CNE-1 cells. By loss-of-function and gain-of-function studies, we observed that over-expression of miR-200a inhibited C666-1 cell growth, migration and invasion, while knock-down of miR-200a enhanced growth, migration and invasion in CNE-1 cells. To understand the molecular mechanisms, we further identified ZEB2 and CTNNB1 as the functional downstream targets of miR-200a to mediate these effects. Interestingly, we found that siRNA suppression CTNNB1 mainly affect cell growth while siRNA suppression ZEB2 mainly affect cell migration and invasion in NPC cells. These data suggest that inhibitory effects of miR-200a on NPC cell growth, migration and invasion are mediated by different targets and pathways. Hence, miR-200a might serve as a regulatory factor of NPC carcinogenesis as well as a potential therapeutic candidate for miRNA-based cancer gene therapy. Keywords: microRNA, Nasopharyngeal Carcinoma, migration, invasion, CTNNB1, ZEB2

880/B38
An Activator of PHD2, KRH 102140, Down-Regulates HIF-1α in Human Cancer Cells.
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Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator that functions as a master regulator of cellular and systemic oxygen homeostasis. HIFs are negatively regulated by a family of prolyl hydroxylases (PHD1-3). The PHD2 isofrom is the main down-regulator of HIFs in normoxia and mild hypoxia, but chemical activators of PHD2 are relatively unknown. Thus, the present study was undertaken to find new activator of PHD2 and to investigate the potential of the compound as a regulator of HIF-1α. Previously, we reported a PHD2 activator, KRH102053 which decreased the protein level of HIF-1α and inhibited angiogenesis. Among derivatives of KRH102053, KRH102140 was found to have more potent than KRH102053 in attenuating HIF-1α. The effects of KRH102140 on controlling HIF were examined in human HOS osteosarcoma, and human HepG2 hepatoma cells. Under our experimental conditions, KRH102140 decreased the protein level of HIF-1α and the mRNA levels of HIF-regulated downstream target genes, which are related to angiogenesis and energy metabolism. Consistent with these results, KRH102140 also significantly inhibited the degree of tube formation in human umbilical vein endothelium cells. These results suggest that KRH102140 have the potential for use as therapeutic agents against various diseases associated with HIF.
HSP70 & HSP90 Promote HSP60 Migration from Cytosol to Mitochondria in Human Colon Cancer.

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Heat Shock Proteins (HSPs), which act as chaperone to refold denatured protein by stress, have been studied to understand cell protection mechanism of various stress responses: Reductive Oxidant Species (ROS), Heat shock, virus infection, cancer and so on. In these conditions, expression of HSPs is remarkably increased. Specially, HSP60 is most abundant protein not only in mitochondria (mtHSP60), but also in cytosol (cyHSP60). While both cyHSP60 and mtHSP60 in normal cells reveal almost equal amount, in stress situation, mtHSP60 is extremely accumulated to protect in mitochondrial matrix and most of cytosol HSP60 quickly immigrates into mitochondria. We found same phenomenon in human cervix, breast and colon cancer in our previous study. Solving to understand these result, we analyzed mitochondrial import system or Transporter Outer Membrane (TOM) complex's mechanism. We obtained normal and colon cancer tissues from four colon cancer patients. We performed co-IP, using non-denature lysis buffer, IgG-A agarose bid, and HSP60 primary antibody and carried out western blot analysis. In results, expressions of cyHSP60-attached HSP70 and HSP90 more increased in colon cancer than normal tissues. The western blot result suggests that cancer cell's cyHSP60 transfer to mitochondria utilizing HSP70 and HSP90. Recently studies have demonstrated that high accumulation of mtHSP60 stabilizes survivin protein which kind of cancer specific gene involved in anti-apoptosis.

Activation of Ras Activity by Grb7 in Cell Migration and Cancer Progression.

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We have previously reported that growth factor receptor-bound protein 7 (Grb7) enable phosphorylated by FAK to facilitate cell migration. More recently, in our laboratory a novel pathway via FAK-Grb7-ERK signaling axis was proposed in cell migration in response to integrin activation. Here, we further demonstrated that Grb7’s RA domain presents a high affinity in association with K-Ras and N-Ras. Moreover, overexpression of wild-type Grb7 was able to promote K-Ras activity but not Y338F mutant, implying that FAK could relay to MAPK cascade via phosphorylating Grb7 and promoting Ras activation in the regulation of cell migration. Consistently, we also found that, under EGF stimulation, wild-type Grb7 can be phosphorylated, enhance cell migration, protect cells from UV-induced apoptosis, and enhance Ras activity. Further studies will elucidate the molecular mechanisms by which Grb7 enables Ras activation and the significance and importance of this novel signaling event in cancer development.

Coronin-1C Expression in Metastatic Melanoma Controls Actin-Dependent Invasive Motility.

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Mammalian Coronin proteins play an important role in regulating F-actin dynamics and cell migration. Based on previous work, type I Coronins (1A, 1B, and 1C) remodel Arp2/3-containing branched actin networks and contribute to disassembly. In addition to interaction with Arp2/3, Coronin 1B is also required for proper targeting of Slingshot-1L, an activating phosphatase for the ADF/cofilin family of actin-binding proteins, to the rear of lamellipodia. Coronin-1C, a closely related isoform, is upregulated in multiple tumor types, and we have identified Coronin-1C as an ERK responsive target that is specifically upregulated in metastatic melanoma. Advanced melanoma is one of the most feared human cancers, and is noteworthy for its therapeutic
resistance, aggressive clinical behavior, and proclivity for early metastasis. Tumor invasion and metastasis utilize multiple actin-dependent processes, including cell migration and invasion, vesicle trafficking, and matrix degradation. Immunofluorescence staining of melanoma cells for Coronin-1C demonstrated that it localizes to structures important for all of these functions: the leading edge of migrating cells, motile cytoplasmic puncta thought to be vesicles, and to invadopodia. To test for functional significance to these processes, we modulated Coronin-1C expression in melanoma cells by overexpression or shRNA-mediated knockdown. We found that shRNA mediated knockdown of Coronin-1C expression in highly aggressive melanoma cells led to decreased cell migration speeds. Furthermore, loss of Coronin-1C decreased invadopodia formation in invasive cells while Coronin-1C overexpression increased invadopodia formation in melanoma cells that initially had low Coronin-1C levels. These data suggest that Coronin-1C promotes the rapid turnover of actin filaments in melanoma cells and increases their capacity to perform actin dependent processes during tumor metastasis. Coronin-1C is therefore at minimum a good biomarker for aggressive melanomas, and potentially of significance when considering the methodology of treatments for these advanced tumors.

884/B42
High Resolution Intravital Imaging Reveals Distinct Tumor Cell Extravasation Pathways Induced by VEGF and Twist Metastatic Genes.
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Metastatic tumor cells invade into the vascular system where they are transported to distant tissues via normal blood circulation until they finally lodge in small capillaries. These cells then extravasate out of the vessel lumen through the endothelium into the surrounding tissues, where they form new tumors. It is controversial, however, as to whether cell extravasation is a regulated and a rate-limiting step during metastasis. Previous work indicated that normal and transformed cells can extravasate equally well independently of their metastatic ability, whereas other data indicates that this process is regulated by specific metastatic gene signatures. Here, we show that cancer cell extravasation is a highly dynamic and complex process regulated by pro-metastatic genes. High resolution time-lapse imaging of vessel-arrested tumor cells revealed that they are not passively immobilized in the lumen as previously believed, but instead display dynamic amoeboid-like movement along the endothelial surface. Surprisingly, cell locomotion can be against or with the blood flow and requires integrin-mediated tumor cell adhesion to the blood vessel wall. Extravasating cells can use para- or trans-cellular modes of emigration and do not damage the vessel wall causing leakage as previously suggested, but rather induce local vessel remodeling characterized by altered endothelial cell-cell junctions and endothelial cell clustering around the invading tumor cell. Tumor cell secretion of pro-angiogenic factor VEGF promoted tumor cell extravasation by increasing vascular wall remodeling in an integrin dependent manner. In contrast, induction of the pro-metastatic gene, twist, caused tumor cells to switch from integrin dependent to an integrin independent mode of extravasation that required ROCK-mediated formation of dynamic membrane blebs and protrusions that penetrated the vascular wall. Our results show that oncogenic gene expression can promote distinct tumor cell extravasation pathways and challenge the current view that extravasation is an unregulated, passive process. These data are vital for establishing tumor cell extravasation as a central target for novel anti-metastatic therapies.

885/B43
MicroRNA-132 Functions as a Novel Angiogenic Switch by Targeting P120RasGAP.
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Activation of the angiogenic program in quiescent endothelial cells is a complex, tightly regulated process during development and disease. We show here that a microRNA, miR-132, functions as
a novel angiogenic switch by targeting p120RasGAP in quiescent endothelium and thereby facilitates angiogenesis. We identified miR-132 during a screen of human microRNAs that were upregulated in HUVECs during growth factor treatment and in a human ES cell model of vasculogenesis. Importantly, miR-132 was also expressed in a majority of human hemangiomas and tumor vasculature but not in normal blood vessels. Transfection of miR-132 decreased endothelial proliferation and tube formation in vitro. Strikingly, injection of anti miR-132 decreased FGF induced angiogenesis in matrigel plugs and developmental angiogenesis in retinas in neonatal mice. Multiple prediction programs identified p120RasGAP as a primary target of miR-132. Expression of miR-132 on HUVECs significantly decreased p120RasGAP levels and Ras activity. p120RasGAP, while highly expressed in normal endothelium, was lost in tumor endothelium in both human and mouse tissues. Moreover, using αVβ3 targeting nanoparticles to deliver anti miR-132 to tumor endothelium, we were able to restore p120RasGAP, suppress tumor angiogenesis and growth in melanoma and breast cancer models. Taken together, we show that miR-132 and p120RasGAP function as a unique angiogenic switch to maintain endothelial quiescence and perturbation of this switch during tumorigenesis is critical for neovascularization.

886/B44
Evaluating the Contribution of 14-3-3 Proteins to Colon Cancer Progression.
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The 14-3-3 class of proteins are a highly conserved family of phospho-threonine-serine binding proteins that contribute to multiple vital cellular functions, including cell cycle regulation, growth, and metabolism. There are seven human isoforms: beta, gamma, epsilon, zeta, eta, theta, and sigma. Previous studies from our laboratory have demonstrated that 14-3-3 proteins affect the cell cycle progression of lung cancer cells and their sensitivity to ionizing radiation. However, only a few reports suggest that 14-3-3 protein expression may be altered in colon cancer, and the majority of these studies used cell culture models. To our knowledge there have been no studies that have assessed the expression of 14-3-3 proteins in the intact colonic epithelium. Therefore, the objective of this study was to determine whether 14-3-3 proteins were expressed in normal mouse colonic epithelium and whether there were any alterations in 14-3-3 protein expression in colon cancer induced by azoxymethane. Using immunohistochemistry with a pan-specific antibody, we determined that 14-3-3 proteins are predominantly expressed in the upper half of the crypt in mouse colonic epithelium. Using antibodies specific to the sigma and gamma isoforms, we determined that 14-3-3 sigma and gamma were also expressed predominantly in the fully-differentiated cells at the top of the crypt. In azoxymethane-induced colon tumors, there was increased and more widespread expression of 14-3-3 proteins. These observations suggest that increased expression of 14-3-3 proteins may contribute to colon cancer progression. Further studies are warranted to determine the specific effects of increased 14-3-3 expression in colon epithelial cells.

887/B45
Genomic Models of Functional Embryomas within Adult Neoplastic Cells.
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Genomic analysis of vertebrate neoplasms often reveals the re-expression of exclusively-embryonic genes within neoplastic adult cells. Exclusively-embryonic genes are normally expressed during embryogenesis, but not again within normal adult cells. Other lifetime-embryonic genes are less constrained, and are co-expressed with adult genes within normal adult cells. The re-expression of one exclusively-embryonic gene within one normal adult cell is sufficient to initiate an adult neoplasm: Okito K, et al, “Generation of germline-competent induced pluripotent stem cells”, Nature 448: 313-317 (July19, 2007). Large embryonic gene networks are often re-expressed intact, as in the epithelial-mesenchymal transition (EMT) mediating

888/B46
Novel Signaling Pathways Linking ErbB2-Mediated Malignancy with Cysteine Cathepsin Activity.

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Almost 1/3 of breast cancer overexpress ErbB2 receptor tyrosine kinase, which is associated with an aggressive disease. Although many of the signaling pathways activated by ErbB2 are well characterized, its downstream effectors that drive breast malignancy are largely unknown. We have analyzed 487 primary breast cancer samples with tissue microarrays or mRNA expression arrays for the expression of ErbB2 and cathepsins B and L and found out that ErbB2 expression correlates with the expression of lysosomal cysteine cathepsins B and L. We show that these two cysteine cathepsins are essential for the ErbB2-induced invasion and aggressive phenotype of breast cancer cells and that their activity can be inhibited by Herceptin treatment. Employing a kinase siRNA screen, we have identified four known mediators of ErbB2 signaling and six previously unknown kinases as crucial regulators of ErbB2-induced cathepsin expression and malignancy and explored their function, hierarchy and their mode of regulation of the malignant cysteine cathepsin activity. Our study indicates that cysteine cathepsins B and L are important mediators of the invasive ErbB2 signaling. Therapeutic interventions targeting the kinases and signaling pathways that ErbB2 utilizes to regulate cathepsin B and L activity may confer clinical benefit in ErbB2-driven malignancies.

889/B47
Human Endothelial Progenitor Cells (EPCs) Role in Tumor Angiogenesis.


Human Endothelial progenitor cells (EPCs) isolated from peripheral blood have the following CD34, VEGFR-2, or AC 133 (CD133) and Aldehyde dehydrogenase antigen-positive cells, which may home to site of neo-vascularization and differentiate into endothelial cells. Endothelial cells contribute to tumor angiogenesis, and can originate from sprouting from neighboring pre-existing vessels. The bone marrow-derived circulating EPCs can contribute to tumor angiogenesis and
growth of certain tumors. In this study we observed EPCs labeled with GFP contribute to breast, brain and prostate cancer tumor angiogenesis in mouse tumor explants. This study confirms the EPCs play a major role in tumor angiogenesis in breast, brain and prostate cancers as indicated in the tumor explants.

890/B48
Human Liver Cancer Stem Cells as a Potential Target for Novel Drug Therapy and Drug Discovery.
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Hepatocellular carcinoma (HCC) is an aggressive tumor with a poor prognosis. Current therapeutic strategies against this disease target mostly rapidly growing differentiated tumor cells. In the present study we have isolated liver cancer stem cells for screening novel drug candidates for the treatment of patients with liver cancer. The metastatic spread of liver cancer cells from the primary tumors to major vital organs, such as lung, colon, brain, and bone, is responsible for the majority of cancer-related deaths. Liver Cancer stem cells are likely to play essential roles in the metastatic spread of primary liver tumors because of their self-renewal capability and their potential to give rise to differentiated progenies that can adapt to different target organ microenvironments. In the present study we have developed a high throughput cell based assay system with human liver cancer stem cells. This assay system has enabled us to identify 300 novel drug candidates for liver cancer patients. The gene expression and protein expression profiles enable one to constructively conclude the novel drugs safety and efficacy. The current cell based assay system enables one to perform novel drug candidates screening with human Liver parental and cancer stem cells simultaneously.

891/B49
Neuromedin-B, Gastrin Releasing Peptide and Bombesin S-3 Receptors in SW-13 and H-295r Human Adrenal Carcinoma Cell Lines.
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Neuromedin-B, gastrin releasing peptide and bombesin S-3 receptors are a closely related seven-transmembrane, G-protein coupled family of receptors which have been strongly conserved during evolution. They are expressed in normal GI and CNS target tissues in humans and have also been shown to be present at high levels in many types of aggressive human malignancies - including cancers of the lung, liver, pancreas, thyroid, and intestine, as well as in neuroectodermal paragangliomas (Bostwick and Bensch, 1985; Price et al., 1985; Schally and Nagy, 2004). There is substantial evidence in the literature showing that antagonists to all three peptide receptors are capable of suppressing the otherwise rapid growth of many of these tumors (Schally and Nagy, 2004). Furthermore, alterations in G-protein coupled receptor genes are believed not only to contribute to the growth of a number of malignancies but also, in some cases, to cell transformation itself and to the induction of a malignant phenotype (Burger et al, 1999). Our objective in this study was to determine whether the bombesin family of receptors exists in human adrenal carcinoma cells. Our results indicate strong transcriptional expression of gastrin-releasing peptide and bombesin S-3 receptor mRNAs using RT-PCR analyses in steroid-producing H-295r human adrenal cortical carcinoma cells cultured in Dulbecco’s Modified Eagles Medium with 10 % fetal calf serum, but very little transcriptional expression of these two peptide receptors in the less differentiated (i.e., mostly non-steroid producing) SW-13 human adrenal cortical carcinoma cell-line. Our studies also indicate that the neuromedin-B receptor is significantly expressed in the SW-13 human adrenal cortical carcinoma cell-line, while being only
weakly expressed in the H-295r cell-line. In conclusion, we see significant but differential expression of the bombesin family of receptors in the SW-13 and H-295r human adrenal carcinoma cell-lines. This work was supported by the U.S. Department of Veterans Affairs and the South Florida Veterans Affairs Foundation for Research & Education. We thank Dr. Jozsef Varga for helpful comments and suggestions.

892/B50
Increased Expression of Human Peptide Deformylase in Certain Cancers.
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Peptide deformylase (PDF) removes the N-formyl group from the initiator methionine in newly synthesized polypeptides in prokaryotes. A human PDF isoform (hsPDF) has been recently discovered that localizes to the mitochondrion, but it is unclear if it plays a role in deformylation of mitochondrial polypeptides. Interestingly, inhibitors of PDF are more cytotoxic toward cancer cells than non-cancer cells, and also display anti-tumor effects in vivo. The objective of this study was to quantify human peptide deformylase in cancerous and non-cancerous tissues. Quantitative real time PCR was used to measure hsPDF mRNA expression in cancerous and non-cancerous tissues of eight different types (breast, colon, kidney, liver, lung, ovary, prostate, and thyroid). HsPDF levels increased in all tumor tissues compared to normal tissues, but increases were most dramatic in breast, colon, and lung tissues where levels were over 3-fold higher in cancer samples. These results suggest that hsPDF is up-regulated in certain cancers, and that hsPDF may play an active role in human cells.

893/B51
DNA Damage after Exposure to Prosthetic Metal Wear Particles.
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The use of Metal on Metal (cobalt-chromium alloy) orthopaedic implants has increased considerably in recent years. It has been shown that as these implants wear away, they generate high quantities of cobalt-chromium particles and ions, which can disseminate to the rest of the body. Exposure to these metal particles has been associated with cytotoxic and genotoxic effects on cells in vitro. To understand better the biological consequences of exposure to prosthetic metal wear particles a mouse model of this exposure has been developed in our laboratory. This model was used to investigate whether exposure to these metal particles causes DNA damage in the bone marrow and brain of treated mice. C3H mice were exposed to metal wear particles (1.2 x 106 µm3/25g mouse; 3µm; approx 4.5 x104). The 2.9 micron cobalt-chromium particles were injected peri-articularly into the right knee of C3H mice at times 0, 6, 12 and 18 weeks. DNA damage 1 week and 1 month after last exposure was determined by comet assay and tail moment was used to evaluate DNA damage. When comparing the tail moment of exposed animals with controls, it was found that exposure to metal particles induced DNA damage in bone marrow 1 week and 1 month after exposure, and no damage was present in brain at any time point. Tail moments in bone marrow cells of the control group after 1 week and 1 month were 1.46 ± 0.12 and 1.98 ± 0.25 respectively, while in the exposed group they were 3.13 ± 0.28 and 4.46 ± 0.68 respectively. Two plausible explanations for these results are i) that ions corroded from the particles may gain access to the bone marrow but not a cross the blood brain barrier; ii) DNA damage by metal In Vivo may depend on rate of cell division. Further studies will be carried out to test these two hypotheses.

894/B52
The Influence of Vanadium Compounds on Human Lung Cancer Cells Line A549.
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In our study we used the cells line A549 obtained from the bank. This is the continuation of the investigation of the vanadium compounds having some ability to normalize the morphological features of neoplastic cells. Control cells. There were mostly cells with lobulated nuclei and highly scant cytoplasm. Sometimes nucleolus undergoing segregation was seen. Pleomorphic mitochondria and scarce rough endoplasmic reticulum canals were observed. The cell membrane was often folded and demonstrated cytoplasmic processes. In general, the cytoplasm did not abounded in organelles. Experimental groups. The cells exposed for 48 hours to the effect of three vanadium compounds: Na3VO4 20mM, NaVO3 20mM and BMOV 40mM - such concentration values were selected, at which approximately 50% cell growth inhibition was determined by biochemical methods MTT and CV. Generally, all the presented cells line A549 from various experimental groups demonstrate changes with a similar character. Only the group BMOV 60 mM of our experiment shows more advanced ultrastructural changes, most likely in consequence of increasing the vanadium dose. This is an effect observed in our previous experiments. on the other hand, metavanadate results in the most severe damage to the cells. The activity of orthovanadate is clearly less effective as compared to metavanadate, however, orthovanadate is less toxic. The observed damage represents typical cytotoxic changes. No such changes have been seen in the controls; dead cells were seen there indeed, but in some percentage of cases, this is a common phenomenon, especially in cell lines cultured in vitro. In the experiment no normalizing effect of vanadium could be detected, neither in particular cell groups nor in the intergroup comparison of vanadium-treated and control cells. These conclusion was based on both biochemical and morphological studies. In all groups it was possible to observe more or less damaged cells under electron microscopy.

895/B53
LKB1 Facilitates Activation of the Unfolded Protein Response in NSCLC Cells.
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Increasing evidence suggests that somatic mutations to the LKB1 tumor suppressor are a common event in the progression of non-small cell lung cancer (NSCLC). LKB1 plays an important role in the regulation of cell metabolism, serving as the primary activator of the metabolic sensor, AMPK. In response to decreases in available ATP, due to nutrient deprivation and/or hypoxia, LKB1-AMPK signaling alters cellular functions in order to restore homeostasis. Loss of LKB1 function and the subsequent failure to activate AMPK during energetic stress conditions, results in cell death. This phenomenon is of clinical interest, as it suggests that induction of energetic stress through pharmacological means might provide significant clinical benefits for patients with LKB1 null NSCLC. We have found that pharmacological induction of energetic stress, using the glucose analog, 2-deoxyglucose (2DG), results in increased toxicity in LKB1 null NSCLC cells. In investigating the mechanisms for 2DG-induced toxicity, we show that LKB1 facilitates activation of the Unfolded Protein Response (UPR), a stress response of the Endoplasmic Reticulum. Microarray analysis revealed that LKB1 expressing NSCLC cells dramatically increased expression of UPR related genes compared to LKB1 null NSCLC cells with 2DG treatment. We provide additional evidence that this novel function is due to LKB1 activity, as re-expression of LKB1 in a LKB1 null NSCLC cells induced expression of UPR markers (CHOP, BiP) in response to 2DG. In conclusion, our study suggests a novel regulatory function of LKB1 and provides insight into the design of therapeutic strategies for LKB1 null NSCLC.

896/B54
Therapeutic Effects of Targeting Integrin A4B1 and Its Mechanism of Action.
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Angiogenesis, the development of new blood vessels, promotes tumor growth and is regulated by integrins, including integrin α4β1. To determine if targeting integrin α4 in tumor models has therapeutic effects, we treated mice bearing subcutaneously injected Lewis lung carcinoma (LLC) tumors and mice that spontaneously develop breast cancer (PyMT model) with a small molecule inhibitor of integrin α4. Mice treated with this inhibitor have significantly decreased tumor volume and weight in the LLC model and decreased tumor burden in the spontaneous breast cancer model. Examination of tumors in the PyMT model shows a significant decrease in both blood vessel and macrophage infiltrates in the treated group compared to the control group. Therefore, targeting integrin α4 has potential for therapeutic value in cancer treatment. To understand the mechanism of how integrin α4β1 is activated we are exploring the role of PI3-kinase α in lymphatic endothelial cells (LECs). We recently found that integrin α4β1 is involved in lymph node lymphangiogenesis, or the development of new lymphatic vessels, of which LEC migration is an essential step. Previous work in our lab showed that PI3-kinase γ activates integrin α4β1 in myeloid cells. However, chemical inhibitors to PI3-kinase α, but not to PI3-kinase γ, block VEGF-C stimulated LEC migration and inhibit In Vivo lymph node lymphangiogenesis. These data suggest that PI3-kinase α regulates LEC migration and potentially activates integrin α4β1 in LECs.

Metabolic Diseases II (897 – 918)

897/B55
Discovering New Biomarkers for Liver Injury.
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Liver disorders are a serious worldwide health issue, but currently liver biopsy remains the main approach for diagnosis, providing information only once the disease is manifested. So, in liver pathology does exist the urgent need of identifying early, sensitive and specific biomarkers detectable by non-invasive techniques that permit us diagnosis, prognosis and monitoring treatments. In this line, our laboratory followed two different approaches, one based on metabolomics and other on proteomics. Firstly, we have conducted a metabolic profile analysis of serum samples from rats treated with D-galactosamine (GalN), a well-established hepatotoxin that causes acute liver damage resembling viral hepatitis. Interestingly, we have found a small subset of metabolites showing strong correlation with the hepatic damage determined by histological analysis of liver sections and by alanine transaminase activity in serum. on the other hand, we have chosen a special kind of extracellular vesicles named exosomes as biological source for biomarker discovery. These vesicles are considered a mirror of the cellular state and constitute a subcellular proteome that can be suitable to identify clinically relevant under-represented proteins. According to that, our group has detected difference in the protein content of hepatocyte-derived exosomes from wild-type and knockout mouse models for chronic hepatic injury, reflecting the changes found in the cell surface of these cells. We have also detected differences in the protein content of exosomes purified from the urine of both chronic (knockout mice) and severe (GalN-treated rats) models regarding the corresponding control animals, pointing out to several putative early and disease progression indicators. Finally, a proteomic analysis of these urinary vesicles in normal conditions revealed the presence of proteins that have been associated to diseases and could be candidate to biomarkers. All these results strongly support the metabolic profiles and exosome proteomics as experimental approaches to identify new specific biomarkers for liver diseases.
898/B56

Potential Novel Treatment for Second-Hand Cigarette Smoke-Induced Liver Steatosis.

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Nonalcoholic fatty liver disease (NAFLD) is the single most common liver problem in the United States. It appears to be linked directly to the growing epidemic of obesity in adults as well as in children. Fatty liver or hepatic steatosis has been classified as the first stage of NAFLD. However, the underlying mechanisms of hepatic steatosis remain unclear. Previously we showed that mice exposed to second-hand smoke, which contains more than 4000 compounds, have lipid accumulation in the liver tissue. This lipid accumulation is caused by modulating the activity of 5'-AMP-activated protein kinase (AMPK) and sterol response element binding protein-1 (SREBP-1), two critical molecules involved in lipid synthesis. We hypothesize that the drug AICAR (5-Aminoimidazole-4-carboxamide ribonucleoside), an AMPK activator, can reverse, at least in part, the lipid accumulation caused by second hand smoke. To test this possibility, we used the ApoB100 transgenic mice fed on high fat diet, exposed them to sidestream whole (SSW) smoke (a major component of “second-hand” smoke), with or without administration of AICAR. We found that in the response to stimulation of SSW for only 5 days, the activity of AMP kinase in the liver was recovered by AICAR, with subsequent inactivation of SREBPs leading to decline in accumulation of fat in the liver. We are currently investigating whether AICAR affects the fatty acid influx, another possible way of accumulation of fat in the liver. This latter process reflects predisposition of individuals to the development of insulin resistance when exposed to smoke. Understanding the regulatory mechanisms of AICAR on the effects of cigarette-smoke-induced steatosis may provide insight into diabetic conditions related to NAFLD and points to possible treatment of these conditions.

899/B57

The Use of Anti-Sense Oligonucleotides to Rescue a Common Mutation in Hermansky-Pudlak Syndrome Type 1.

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Hermansky-Pudlak syndrome (HPS) is due to defects in the biogenesis of lysosome-related organelles, such as melanosomes in melanocytes and delta granules in platelets. As a result, clinical features of HPS patients include ocuocutaneous albinism, a bleeding diathesis, and other sporadic complications, such as granulomatous colitis or a fatal pulmonary fibrosis. The most common subtype, HPS-1, occurs primarily among Puerto-Ricans and results from a 16-bp duplication founder mutation in exon 15 of the HPS1 gene. Little is known about the function and structure of HPS1, making directed therapy difficult. In view of successful advances in therapeutic exon skipping (e.g., for Duchene Muscular Dystrophy), we introduced anti-sense morpholino oligonucleotides (MOs) to HPS patients’ cultured melanocytes to induce skipping of in-frame exons carrying deleterious HPS1 mutations. The effects and efficacy of each MO on the HPS1 mRNA transcript were validated using RT-PCR analysis. Furthermore, we completed immunofluorescence (IF) staining against the melanosome-specific protein TYRP1 to detect the correction of abnormal melanosome trafficking patterns defects (i.e. lack of accumulation in dendritic tips) that are typical for HPS-1. Exon 12, exon 13, and exon 15 MOs effectively removed ~75%, 95%, and 60%, respectively, of their targeted exon in HPS1 transcripts. IF analysis of patients’ melanocytes transfected with exon 12 MO and exon 13 MO did not rescue the lack of melanosomal accumulation at the dendritic tips; however, this staining appeared to be increased in patients’ melanocytes transfected with the exon 15 MO. Ongoing immunoblot assays against HPS1 and HPS4, which interact in the Biogenesis of Lysosome-related Organelles Complex (BLOC)-3, will further verify cellular functionality of these exon-skipped HPS1 isoforms. Further investigations into targeted exon skipping could result in clinical applications for the treatment of systemic complications associated with HPS-1. More specifically, the promising results of skipping of exon 15 may prove to be extremely beneficial for the large group of Puerto-Rican HPS-1 patients with the 16-bp duplication founder mutation.
900/B58
Divalent Interaction between HPS1 and HPS4 to Form the Biogenesis of Lysosome-Related Organelles 3 (BLOC-3).
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Hermansky-Pudlak Syndrome (HPS) [MIM#203300] is a rare autosomal recessive disorder characterized by oculocutaneous albinism, a bleeding tendency, and sporadic pulmonary fibrosis, granulomatous colitis or infections. HPS is a heterogeneous disorder; eight HPS-causing genes have been identified in humans. HPS type 1 is the most severe subtype with a prevalence of ~1/1800 in Northwest Puerto Rico. Mutations in HPS genes affect the biogenesis of lysosome-related organelles such as melanosomes and platelet dense granules. The products of the HPS type 1 and 4 genes (i.e., HPS1 and HPS4) assemble to form a complex known as Biogenesis of Lysosome-related Organelle Complex 3 (BLOC-3). Here, we report the identification of two interacting regions in each HPS protein required to form the BLOC-3. We found that HPS1 interacts with HPS4 in two regions, spanning amino acids 1-197 and 506-700 but not with the middle portion of HPS1 (250-505). In addition, HPS4 displays dual binding sites to HPS1, comprised by its N-terminus (1-230) and a distinct region between residues 340-399. Further interaction studies showed that the N- termini of HPS1 and HPS4 interact with each other and that a discrete region of HPS4 (340-708) showed interactions with both the N- and C- termini of HPS1 protein. However, the C- terminus of HPS4 (528-708) by itself did not show any interaction with HPS1, suggesting no participation in the formation of the HPS1-HPS4 complex. In Vitro assays with truncated HPS1, mimicking mutations found in HPS1 patients, showed retention of some interaction with HPS4, suggesting the possibility to generate (a truncated form of) BLOC-3 in these patients that could retain partial function and could possibly explain the variable progression of the pulmonary fibrosis in BLOC-3 deficient patients. These observations not only extend our understanding of BLOC-3 assembly, but also represents an important first step in the mapping of the domains that may sense regulatory signals responsible for the biogenesis of lysosome-related organelles. This work was partially supported by the IRTA-NIH Program and grants R25GM81838 and RCMI G12RR03051.

901/B59
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Congenital disorders of glycosylation (CDG) are a rare group of inherited metabolic disorders caused by defective glycosylation of proteins. Though CDG patients present variable clinical phenotypes, however they show a common feature of deglycosylation of proteins. Recent studies have provided some insight into the basic mechanisms associated with pathogenesis of CDG. We have investigated the mitogenic activity of Insulin (Insulin-Transferrin-Selenium, ITS) and Insulin-like growth factor-1 (IGF-1) in peripheral blood lymphocytes isolated from CDG patients. Cultures of lymphocytes were established from blood samples of six confirmed patients of CDG and twelve age and gender matched healthy controls. Cells were serum-starved for 36 hrs before treatment with varying concentrations of insulin and IGF-1. Mitogenic activity of growth factors was determined by measuring the DNA synthesis in cell cultures in the presence / absence of ITS/IGF-1. We observed that lymphocytes isolated from CDG patients had significantly (p< 0.001) reduced DNA synthesis in response to a 24 hr treatment with IGF-1 (50ng/ml) as compared to IGF-1-induced DNA synthesis in lymphocytes isolated from controls. Interestingly, lymphocytes from CDG patients and normal subjects did not show marked difference in their DNA synthesis following treatment with Insulin. In conclusion, our results show that CDG patients have impaired mitogenic response to IGF-1, however insulin-mediated DNA synthesis remains unaffected by deglycosylation of proteins in these patients. ( Funded by research grant MK 01 /05).
902/B60
Mechanical Stretch Enhances MMP-2 Production in Vascular Smooth Muscle via Activation of Akt Pathway.
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Increased biomechanical stresses in the fibrous cap of atherosclerotic plaques contribute to plaque rupture, and increased risk of symptomatic plaque rupture seems to be partially mediated by matrix metalloproteinases (MMP). However, the role of mechanical strain on MMP production in vascular smooth muscle is still unclear. Therefore, this study determined the role of mechanical stretch on MMP production, and signal pathways involved in mechanical stretch-induced MMP production in VSMC. When VSMC was stimulated with cyclic stretch (0-10% strain, 60 cycles/min, 0-24 hrs, sine waveform) using Flexcell Tension Plus FX-4000T system, both the production and gelatinolytic activity of MMP-2, but not MMP-9, were increased in a frequency- and time-dependent manner. Among the possible VSMC signaling pathways modulated by stretch, phosphorylation of Akt and ERK, but not p38MAPK and JNK was increased by 10% stretch in a time-dependent manner to 240 min. Stretch-induced MMP-2 production with increased activity was exclusively decreased by inhibition of PI3K/Akt pathway, but not by inhibition of other MAPK pathways including ERK pathway. Collectively, our data indicate that mechanical stretch induces MMP-2 production in VSMC, and it is mediated at least partly by activation of the PI3K/Akt pathways, consequently leading to atherosclerotic plaque instability. (this study will be included the effect of signal specific isoform for Akt by short hairpin RNA knockdown approach.)

903/B61
Acrolein Enhances 5-Lipoxygenase Expression and Activity on Murine Macrophages via EGF-Receptor and ERK Pathways.
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Oxidation of lipids is considered a key feature of atherogenesis. Acrolein, a major component of lipid peroxidation product, has been implicated in the development of atherosclerosis. 5-lipoxygenase (5-LO) in atherosclerosis lesions was mainly localized in macrophages. However, the pathogenic role of acrolein in macrophages responses has not yet been elucidated. Therefore, we investigated the role of acrolein on 5-LO expression and activity in murine macrophages. Stimulation of J774A.1 cells with acrolein led to 5-LO mRNA and protein expression in association with 5-LO activity, as measured by leukotriene B4 (LTB4) production in a concentration- and time- dependent manners. Among the mitogen-activated protein kinase (MAPK) pathways, acrolein increased phosphorylation of ERK, but not JNK and p38 MAPK up to 120 min. Linked to these results, the increased 5-LO expression and activity by Acrolein were significantly attenuated by PD98059, an ERK inhibitor, suggest a role of ERK signaling on 5-LO expression and activity by acrolein. Moreover, Acrolein-induced 5-LO expression was blunted by AG1478, an epidermal growth factor receptor (EGFR) inhibitor, but not by AG1295, a platelet derived growth factor receptor (PDGFR) inhibitor, confirming that AG1478, a EGFR inhibitor prevented increased phosphorylation ERK by acrolein. Collectively, these data suggest that Acrolein-induced 5-LO expression and activation is activation of EGFR mediated ERK pathways, consequently leading to development and progression of atherosclerosis.

904/B62
NF-xB and Sp1 Contributes 4-Hydroxynonenal-Mediated Up-Regulation of 5-Lipoxygenase Expression in J774A.1 Cells.
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Increased levels of 4-hydroxynonenal (HNE) and 5-lipoxygenase (5-LO) co-exist in macrophages in atherosclerotic lesions. While 5-LO has been suggested as a modulator of atherosclerotic
plaque instability, it remains unclear on the mechanism by which it is regulated. To gain insights of transcriptional regulation of 5-LO gene, we examined the promoter activity of 5-LO. We have amplified a genomic sequence of promoter 2.0 kb upstream of the transcription initiation site of mouse genomic DNA by PCR. A series of sequentially deleted fragments were fused to pGL3 vector containing a luciferase reporter gene. Luciferase assay was performed by transfecting the constructs into a murine macrophages cell line, J774A.1. The assay resulted in the identification of promoter region 50-213 bp upstream of transcription start site, which is responsible for a remarkable enhancement of transcriptional by HNE. A site-directed mutagenesis of this region revealed that transcriptional factors including Sp1 and NF-κB were associated with up-regulation of HNE-induced 5-LO expression. The role of Sp1 and NF-κB in 5-LO expression was further confirmed by siRNA knockdown of Sp1 and NF-κB. Importantly, among the MAPK pathways, ERK inhibitors, PD98059 and p38 MAPK inhibitors, SB203580 were attenuated HNE-induced NF-κB and Sp1 activity. Collectively, these data suggest a differential requirement that ERK/NF-κB and p38 MAPK/Sp1 pathway were up-regulated 5-LO expression by HNE, consequently provide novel options for therapeutic interventions to regulate 5-LO expression in atherosclerosis.

905/B63
Functional Significance of a Single Nucleotide Polymorphism in Neuropeptide Y Receptor 2 Gene Candidate Gene for Hypertension.
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Hypertension (HTN), or high blood pressure, affects 1 in 3 adults in the US and is an important risk factor for cardiovascular and kidney diseases. Multiple genes as well as environment and lifestyle factors influence the risk of developing high blood pressure. Our previous work in animal models and human populations identified Neuropeptide Y receptor 2 (NPY2R) as a candidate gene for hypertension. In two independent Japanese populations, bearing the GG genotype at a single nucleotide polymorphism (SNP) located 224 bp upstream of the transcription start site of NPY2R (-224 A>G) was associated with a protective effect against HTN. However, the functional significance of the NPY2R -224 A>G SNP and its potential role in the HTN at the molecular level remains unknown. Our objective was to assess the effect of the a to G substitution in the promoter of NPY2R on the expression levels of this gene. Human Embryonic Kidney (HEK) 293 cells were transfected with pGL4.10 DNA constructs containing the promoter of NPY2R and each nucleotide a or G at the -224 position upstream of the luciferase reporter gene. Interestingly, the luciferase activity induced by the NPY2R promoter with the G allele in position -224 was reduced by 34% compared to that induced by the promoter bearing an A. The different levels of relative Luciferase activity between a and G constructs were statistically significant (p < 0.01). Together, our data provide strong evidence for a functional role of NPY2R in genetic predisposition to hypertension.

906/B64
Anti-Hypertensive Effect of Gomisin a in Angiotensin II-Induced Hypertensive Mice.
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Gomisin a (GA) is a small molecular weight lignan contained in Fructus Schisandrae, the dried seed of schisandra chinensis which is widely used as a tonic in traditional Korean medicine. Previously, we demonstrated that GA induced vascular relaxation through partially activation of endothelium-dependent nitric oxide pathway, and partially dephosphorylation of myosin light chain. In this study, we examined the anti-hypertensive effects of GA in angiotensin II(AngII)-induced hypertensive mice. C57BL6 mice infused subcutaneously with AngII (2μg/kg/min, osmotic mini-pump) showed an increase in blood pressure. To determine the single-dose effect, GA was infused into femoral vein, and then blood pressure was monitored via carotid artery. at
concentrations of 10 μg/kg - 100 μg/kg, blood pressure in hypertensive mice, but not in control mice, decreased by GA in a concentration-dependent manner. In the continuous-dose test, GA (2 and 10 μg/kg/min) was infused subcutaneously for 2 weeks using osmotic pump. The increase in blood pressure in AngII-infused mice was significantly attenuated by infusion of GA in a dose-dependent manner, while GA had no effect on blood pressure in control mice. Based on these results with our previous study in which GA directly induced vasorelaxation, it is suggested that GA has anti-hypertensive effects via its direct effects on hypertensive vasculature. However, further experiments are needed to determine the signal pathways involved in GA-mediated anti-hypertensive effects.

907/B65
Expression of Usher Syndrome Type 3 -Gene (CLRN1) in Zebrafish Sensory Cells.
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Objective: Usher syndrome (USH) is the most common form of combined blindness and deafness. Usher syndrome type 3 (USH3) is the rarest subtype caused by mutations in Clarin 1 -gene (CLRN1). CLRN1 protein has four predicted transmembrane regions and is known to localize in the plasma membrane when expressed in cell cultures. The function of CLRN1 in the retina and the cochlea remains unknown. In this study, we investigate the expression and localization of clrn1 in zebrafish in order to enlighten the function of clrn1 in hearing, balance and sight. Methods: The main zebrafish clrn1 variant expression was studied from sectioned zebrafish adult and larvae eyes and ears with in situ hybridization. The Clrn1 protein localization was studied with antibody specific to zebrafish Clrn1. Morpholino oligonucleotide (MO) specific to clrn1 was used to inhibit clrn1 expression and the functional and morphological effects of Clrn1 absence were studied with behavioural tests and histological studies. Results: The in situ hybridization studies showed zebrafish clrn1 expression in hair cells of the larvae ear and neuromasts. In larval retina the in situ expression was concentrated in the inner nuclear layer (INL), in adult retina clrn1 expression was also found in the photoreceptors. The Clrn1 antibody studies showed similar localization dynamics with the Clrn1 first appearing in the INL in larval retina and in adult also in the photoreceptors. When Clrn1 production was inhibited with a clrn1 translation blocking morpholino, the injected larvae showed reduced levels of protein in the retina, exhibited slower optokinetic responses (OKR) and had obvious balance problems. Conclusions: Zebrafish clrn1 expression and protein localization in ear hair cells was consistent with the reported findings of Clrn1 expression in mouse cochlea. In the retina, it seems Clrn1 is present both in the adult INL and photoreceptors. Our studies showed that both clrn1 RNA and protein are present in the zebrafish larval ear and eye, and blocking clrn1 translation and thus presence of Clrn1 protein produces defects in vision, hearing, and balance.

908/B66
Modified Mild Heat Shock with Electrical Stimulation Ameliorates Progressive Proteinuria in Mouse Model of Alport Syndrome.
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Alport Syndrome (AS) is a hereditary nephritis caused by defects in the genes encoding alpha-3, alpha-4, or alpha-5 chains of type IV collagen (COL4A3, COL4A4, COL4A5, respectively) in the glomerular basement membrane. The estimated gene frequency ratio of AS is 1:5000. Among AS patients, X-linked AS (XLAS) is the most common disease, which is caused by mutations in COL4A5 gene in the X chromosome and accounts for 85% of the cases. Approximately 90% of patients with AS develop end-stage renal disease (ESRD) by age 40 years. Despite the importance of AS, no effective treatment yet exists for AS patients. Novel therapeutic strategies
are thus urgently needed for progressive proteinuria and nephritis in AS patients. In the present study, we show that combination treatment with mild electrical stimulation (MES) and heat shock (HS) ameliorates progressive proteinuria and albuminuria in XLAS mouse model. We also demonstrate that MES with HS prevents the progressive nephritis in Alport mice. Interestingly, the activation of 5'-AMP activated protein kinase (AMPK), a crucial factor for regulating proteinuria, was seen in the kidneys of mice treated with MES+HS. Although the molecular mechanisms of how MES with HS ameliorates proteinuria and activates AMPK need to be further elucidated, our study provides a novel therapeutic strategy for Alport syndrome.

909/B67
Diabetes Induced Glomerular Filtration Barrier Damage Is Ameliorated by Podocyte Targeted Metallothionein Overexpression in OVE26 Mice.
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The OVE26 transgenic diabetic mouse exhibits several characteristics of human diabetic nephropathy (DN), including glomerular and mesangial hypertrophy, increased glomerular apoptosis, increased tubular albumin and severe albuminuria. To test our hypothesis that diabetic renal injury may be the result of oxidative insult in the glomerular filtration barrier, Nmt transgenic mice that overexpress the antioxidant protein metallothionein (MT) specifically in podocytes were crossed with OVE26 diabetic mice. The effects of podocyte MT overexpression on glomerular basement membrane (GBM) thickness, glomerular volume, glomerular cell density and number, podocyte effacement, and endothelial cell fenestration were analyzed by TEM morphometry in 150 day-old OVE26, Nmt, OVE26/Nmt, and FVB (control) mice. Urinary albumin excretion (UAE) was measured by ELISA. Compared to normoglycemic Nmt and FVB controls, OVE26 diabetic mice showed significantly increased GBM thickness, glomerular volume, mesangial and endothelial cell proliferation, total glomerular cell number, and UAE. Podocyte density was decreased, though absolute numbers were similar in diabetics and controls. Importantly, OVE26/Nmt mice exhibited significant protection against diabetes-induced increased GBM thickening, glomerular hypertrophy, mesangial and endothelial cell proliferation and UAE. While podocyte numbers remained unchanged, podocyte damage (effacement) and endothelial cell injury (substantial fenestration loss) were significantly attenuated in OVE26/Nmt mice. These results provide evidence for a direct role of oxidative damage to the podocyte in diabetic glomerular filtration barrier dysfunction, and show that podocyte specific MT overexpression can reduce or delay several primary complications of DN. Funded in part by NIH Grant DK072032.

910/B68
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HIBM is an adult-onset, progressive neuromuscular disorder, caused by GNE mutations. GNE encodes the ubiquitously expressed, key enzyme in sialic acid (SA) synthesis, UDP-GlcNAc 2-epimerase/ManNAc kinase. We created an HIBM mouse model, mimicking the Persian-Jewish founder mutation M712T. Mutant mice unexpectedly died before day 3 of life (P3) from severe glomerulopathy due to hyposialylation, but survive with treatment if fed the SA precursor ManNAc. In addition to administering precursors in the SA pathway, we sought to deliver an intact GNE gene as a feasible treatment option. We created a human non-viral GNE-plasmid embedded in cationic liposomes (hGNE-lipoplex), which was administered via intramuscular injection (biceps and extensor carpi radialis longus) to one HIBM patient. The patient showed no adverse effects and her arm muscle strength improved moderately. To proceed with hGNE-
lipoplex intravenous (IV) or intrahepatic (the major organ of SA synthesis) injections in patients, we tested these treatments in our HIBM mice. hGNE-lipoplex was injected IV (retro-orbitally) and intra-hepatically in litters at age P1. Survival beyond P3 was the initial outcome parameter. The major tissues of treated mice were assessed at P5 for hGNE expression, glomerular disease, sialylation, and histology. Intrahepatic treatment yielded survival of all wild type (+/+) and heterozygous (+/-) pups, but just one of 8 mutants survived beyond P3; that pup had increased sialylation of the glomerular glycoprotein podoclyxin at P5. IV injections resulted in no surviving mutants beyond P3; however, some +/- pups died as well, indicating possible lethality of the injection method. Surviving +/+ showed hGNE expression in liver and kidney at P5, indicating no toxicity of hGNE-lipoplex and effective gene delivery to these tissues. It is possible that hGNE-lipoplex treatment at P1 (untreated mice die at P3) may not allow enough time for hGNE protein to be expressed and to produce enough SA, whereas the ManNAc treatment immediately delivers the substrate, bypassing the critical GNE processing step, allowing for faster production of SA. Further animal studies are planned to elucidate the timing and efficacy of GNE-lipoplex therapy.

911/B69
Sialic Acids Prevent the Myopathic Phenotype in the DMRV/hIBM Mouse Model.
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Distal myopathy with rimmed vacuoles (DMRV) or hereditary inclusion body myopathy (hIBM) is a progressive autosomal recessive myopathy affecting adult patients. This debilitating myopathy, for which up to this time has no cure, renders patients non-ambulatory about 12 years after the disease onset. Characteristic findings include skeletal muscle atrophy and weakness, and accumulation of autophagic vacuoles in myofibers, scattered atrophic fibers, and intracellular accumulation of amyloid and other proteins. The development of therapy for DMRV/hIBM has primarily been hampered by the lack of understanding of disease pathomechanism. Although this myopathy is secondary to mutations in the UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) gene that is crucial in the synthesis of sialic acid, it has been controversial whether hyposialylation contributes to the pathogenesis of DMRV/hIBM. Nevertheless we recently shown that DMRV/hIBM mouse model, a Gne deficient mouse that expressed the human GNE p.D176V mutation, hyposialylation was documented in serum and various organs, in addition to the characteristic features found in the human disease. Here, we evaluated the effect of sialic acid metabolites in the DMRV/hIBM mice. After determining the efficient route and dose of administering these metabolites, we gave three types of agents (20 mg/kg/day): ManNAc, the physiologic sialic acid precursor; NeuAc, the most abundant sialic acid; or sialyllactose, a sialic acid conjugate. All agents were continuously given from 10-20 weeks of age until the mice reached 55-57 weeks. Analysis of motor performance and ex vivo muscle contractile properties showed a remarkable improvement in all parameters tested. Muscle atrophy and myofiber degeneration were remarkably reduced. These findings were accompanied by an increase in sialic acid levels in the muscle, serum, and other organs. These data provide evidence that hyposialylation plays a major role in disease pathomechanism. Moreover, our results demonstrate a promising strategy that can potentially be used in the treatment of DMRV/hIBM.

912/B70
Muscle Atrophy in the DMRV/hIBM Mouse Model.
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Distal myopathy with rimmed vacuoles (DMRV), also called hereditary inclusion body myopathy (hIBM), is an autosomal recessive debilitating disorder affecting young adults with the age of onset ranging from 15 years to late thirties. The disease is characterized clinically by preferential involvement of tibialis anterior and hamstring muscles and relative sparing of quadriceps, and
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pathologically by the presence of rimmed vacuoles, which are seen on electron microscopy as clusters of autophagic vacuoles, in addition to scattered atrophic fibers and muscle degeneration. Up to this time, treatment for this myopathy remains elusive. DMRV/hIBM is caused by missense mutations in GNE gene that encodes the essential enzyme in sialic acid biosynthesis. We recently generated a model mouse for DMRV/hIBM that expressed human GNE with the missense mutation D176V, but lacks the endogenous mouse GNE. This DMRV/hIBM model exhibited hyposialylation in serum and various organs which predated the skeletal muscle weakness, atrophy, rimmed vacuole formation, and deposition of amyloid and various proteins within the myofibers, supporting the theory of hyposialylation in the pathomechanism of DMRV/hIBM. In this study, we analyzed chronological changes in muscle size, myofiber cross-section area and pathological alteration of the gastrocnemius muscles of the DMRV/hIBM mice. Myofiber atrophy was noted in all fiber-types from 20 weeks of age, which preceded and was independent from the characteristic hallmarks on pathology, including amyloid deposition and rimmed vacuoles at 50 weeks of age. In addition, muscle atrophy was accompanied by upregulation in the expression of autophagy-related genes and atrogenes that encode ubiquitin ligases. These data suggest that the activation of lysosomal and proteosomal degradation pathways in muscles may contribute to myofiber atrophy in the DMRV/hIBM mouse model.

913/B71
Locally Acting IGF-1 Isoform (mIGF-1) Protects Cardiomyocytes from Hypertrophic and Oxidative Stresses via SirT1 Activity.
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Oxidative and hypertrophic stresses contribute to the pathogenesis of heart failure. Insulin growth factor-1 (IGF-1) is a peptide hormone with a complex post-transcriptional regulation, generating distinct isoforms. We have previously shown that a locally acting IGF-1 isoform (mIGF-1) helps the heart to recover from toxic injury and from infarct. Sirtuin 1 (SirT1) belongs to the sirtuin family of NAD+-dependent protein deacetylases, whose activation lengthens life span and could prove beneficial for human health. In the murine heart, moderate SirT1 overexpression was reported to mitigate oxidative stress. Circulating IGF-1 isoform and SirT1 play antagonizing roles in life span determination and share molecular downstream targets in the heart, in turn affecting cardiomyocyte physiology. However, how different IGF-1 isoforms may impact SirT1 and affect cardiomyocyte function is unknown. Here we show that, unlike circulating IGF-1 isoform, locally acting mIGF-1 increases SirT1 expression/activity in cultured HL-1 and in neonatal mouse cardiomyocytes. mIGF-1-induced SirT1 activity exerts protection against angiotensin II (Ang II) -triggered hypertrophy. Moreover, we found that SirT1 is required for mIGF-1-mediated protection against paraquat (PQ) and Ang II-induced oxidative stress, while circulating IGF-1 isoform was unable to do so. Interestingly, potent cardio-protective genes (adiponectin, UCP-1 and MT-2) were increased in mIGF-1-overexpressing cardiomyocytes, in a SirT1-dependent fashion. Conversely, circulating IGF-1 isoform did not affect adiponectin, UCP-1 and MT-2 mRNA levels. Thus, mIGF-1 protects cardiomyocytes from oxidative and hypertrophic stresses via SirT1 activity, and may represent a promising therapeutic target to fight heart diseases.

914/B72
O-Glcnacylation Regulates Hyperglycemia-Induced GPX1 Activation.
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The antioxidant enzyme glutathione peroxidase 1 (GPX1) has an important role in cell survivals in response to oxidative stress. It is well known that oxidative stress-induced phosphorylation of
GPX1 regulates its activity. Also, hyperglycemia-induced GPX1 activation has been reported, but the exact mechanism has not been established yet. In this study, we show that posttranslational modification of GPX1 by O-linked N-acetylglucosamine (O-GlcNAc) increases under hyperglycemic conditions and the modification occurs at the C-terminus of GPX1. And we investigate that O-GlcNAcylation of GPX1 increases its binding to the c-Abl/Arg tyrosine kinases, which are important for oxidative stress-induced GPX1 activation, resulting in increased GPX1 activity. Furthermore, we demonstrate that NAG-thiazoline (NTZ), O-GlcNAcase inhibitor, treatment can induce GPX1 activation in the mice tissue. Our findings suggest that O-GlcNAcylation has an important role in GPX1 activation under hyperglycemic conditions due to diabetes.

915/B73
Creation of a Mouse Model for Lowe Syndrome.
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Lowe syndrome is an X-linked disorder characterized by congenital cataracts, intellectual deficits, and renal tubular dysfunction characterized by low molecular weight (LMW) proteinuria, renal tubular acidosis, aminoaciduria, phosphaturia, and inability to concentrate urine. It is caused by loss-of-function mutations in the OCRL1 gene, a PIP2 5-phosphatase. Mutations in OCRL1 are also responsible for a subset of Dent disease, a renal disorder characterized by LMW proteinuria, hyperphosphatemia, and hypercalciuria. To date, a mouse model of Lowe syndrome has not been available, as mice deficient in either Ocr1 or Inpp5b, an autosomal paralog of Ocr1, do not recapitulate the disease. Mice deficient in both Ocr1 and Inpp5b die prior to implantation, indicating functional overlap. We hypothesize that Inpp5b may compensate completely for the loss of Ocr1 in mice, while INPP5B may only partially compensate in humans, leading to Lowe syndrome. To develop a model of Lowe syndrome, we set out to replace mouse Inpp5b with human INPP5B in Ocr1 knockout mice. First, we established transgenic mouse lines expressing human INPP5B from a bacterial artificial chromosome. We crossed these lines with Inpp5b-/- mice and subsequently with Ocr1-/- or Ocr1-/-Y mice. Double knockout mice expressing one copy of human INPP5B (Inpp5b-/-; Ocr1-Y; BAC-Tg-) showed reduced post-natal growth and substantial LMW proteinuria. A subset of these mice was severely runted, died prematurely, and had larger amounts of LMW proteinuria. Double knockout mice expressing two copies of human INPP5B (Inpp5b-/-; Ocr1-Y; BAC-Tg+) also have reduced post-natal growth, but only small, variable amounts of LMW proteinuria, and no severe runting. Mice with one or two copies of mouse Inpp5b who are deficient in Ocr1 and do not express human INPP5B have normal growth and no LMW proteinuria. We conclude that mouse Inpp5b prevents the tubular abnormalities caused by the loss of Ocr1 whereas human INPP5B does not, and that there is a gene dosage effect in the degree of compensation by human INPP5B.

916/B74
Defining the Role of OCRL1 in Endocytic Trafficking in the Search for Drug Targets in Lowe Syndrome.
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OCRL1 is a phosphatidylinositol-4,5-bisphosphate 5-phosphatase (PI5P5ase) that cycles between the endosomal compartments and the Golgi complex. Mutations in the ocr1 gene cause Lowe syndrome, a rare X-linked disease that is characterised by congenital cataracts, central hypotonia and renal Fanconi’s syndrome (low molecular weight proteinuria, tubular acidosis). OCRL1 is a multidomain protein with a split N-terminal PH domain, a central 5-phosphatase catalytic domain,
an ASH domain, a C-terminal catalytically inactive Rho GAP domain, multiple clathrin binding motifs, and Rab binding regions. Through these domains and motifs, OCRL1 interacts with key components of the membrane trafficking machineries, such as AP2, clathrin, Rab5, Rab6, Rab14, Arf6 and APPL1. Rather surprisingly, our knowledge of the actual functional role of OCRL1 lags well behind that of its molecular structure and interactors. To gain insight into this and into its PI5Ptase activity in membrane trafficking and in the pathogenetic mechanisms of Lowe syndrome, we undertook two independent approaches: analysis of the endocytic compartments in kidney proximal tubular cells obtained from Lowe syndrome patients; and study of the multiple endocytic pathways and the functional and ultrastructural consequences of the knock-down of OCRL-1 (by RNA interference). Our results indicate that through its PI5Ptase activity, OCRL maintains the identity and function of the early endosomes (through control of their lipid and protein compositions), the structure of the early endosomes as a compartment, and the efficiency of the trafficking pathways that traverse this compartment. This includes megalin, the multiligand receptor that drives the uptake and absorption of proteins in proximal tubular cells, and that undergoes mistrafficking in cells devoid of functional OCRL-1. We have exploited the endocytic defects of cells deprived of OCRL-1 to set up robust and reproducible assays to be used in high-content screening of siRNA libraries or small molecule libraries to identify drug targets and hit compounds for drug development.

917/B75

Lowe Syndrome Patient Fibroblasts Display OCRL1-Specific Cell Migration Defects That Cannot Be Rescued by the Homologous Inpp5b Phosphatase.
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The Lowe syndrome is an X-linked, life-threatening, developmental disease characterized by mental retardation, cataracts and renal failure. Although this human illness has been linked to defective function of the phosphatidylinositol 5-phosphatase, Ocr1 (Oculo-Cerebro-Renal syndrome of Lowe protein 1), the mechanism by which this enzyme deficiency triggers the disease is not clear. Ocr1 is known to localize mainly to the Golgi apparatus and endosomes, however it translocates to plasma membrane ruffles upon cell stimulation with growth factors. The functional implications of this inducible translocation to the plasma membrane are presently unknown. Here we show that Ocr1 is required for proper cell migration, spreading and fluid-phase uptake in both established cell lines and human dermal fibroblasts. In agreement with these results, we found that primary fibroblasts from two patients diagnosed with Lowe syndrome displayed similar cellular defects. Importantly, these abnormalities were suppressed by expressing wild-type Ocr1 but not by a phosphatase-deficient mutant. Interestingly, the homologous human PI-5-phosphatase, Inpp5b, was unable to complement the Ocr1-dependent cell migration defect. Further, Ocr1 variants that cannot bind the endocytic adaptor AP2 or clathrin, like Inpp5b, were less apt to rescue the migration phenotype. However, no defect in membrane recruitment of AP2/clathrin or in transferrin endocytosis by patient cells was detected. Collectively, our results suggest that Ocr1, but not Inpp5b, is involved in ruffle-mediated membrane remodeling. Our results provide new elements for understanding how Ocr1 deficiency lead to the abnormalities associated with the Lowe syndrome.

918/B76

Role of OCRL1 in Renal Epithelial Membrane Traffic.
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Lowe Syndrome is an X-linked disease resulting from mutations in OCRL1, an enzyme that hydrolyzes the 5’-phosphate from phosphatidylinositol 4,5-bisphosphate (PIP2). Lowe Syndrome patients develop Fanconi syndrome, a renal disorder characterized by urinary loss of excess amounts of low molecular weight proteins, amino acids, bicarbonate, minerals, and phosphate.
Proteinuria is thought to result from impaired function of the multiligand receptor megalin in the kidney proximal tubule. Because PIP2 modulates numerous steps in endocytosis, it has been suggested that OCRL1 deficiency causes impaired megalin trafficking resulting in proteinuria. To test this hypothesis, we knocked down OCRL1 in canine and human renal epithelial cells using siRNA. Cellular PIP2 levels were slightly elevated in cells depleted of OCRL1, and we measured an increase in actin comet frequency in these cells, consistent with previous observations in primary fibroblasts cultured from Lowe Syndrome patients. Apical biosynthetic delivery kinetics were unaffected by OCRL1 knockdown, indicating that the population of PIP2 modulated by OCRL1 is likely not involved in the trafficking of newly synthesized proteins. We did not observe defects in megalin endocytosis or in the fate of megalin ligands in cells lacking OCRL1. Delivery of newly synthesized lysosomal hydrolases, however, was impaired in these cells, suggesting that OCRL1 plays a role in membrane trafficking between the TGN and endosomes. Together, our studies indicate that OCRL1 is not directly involved in receptor mediated endocytosis and megalin ligand uptake. Rather, the renal symptoms observed in Lowe Syndrome patients are likely to result from downstream effects due to loss of OCRL1 function.

Neuronal Diseases I (919 – 939)

919/B77
Generation of Transgenic Zebrafish Expressing Cytotoxic Beta-Amyloid (Aβ42) Peptides as a Novel Alzheimer's Disease Model.
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Beta-amyloid peptide (Aβ42) is a proteolytically generated short peptide from amyloid precursor protein (APP), and plays a vital role in the pathogenesis of Alzheimer’s disease (AD) due to cytotoxicity stemming from its oligomerization. In contrast to previously established AD animal models where the uncleaved mutant APP from human AD patients is expressed, our approach takes advantage of the direct expression of the toxic human Aβ42 (hAβ42) peptides using transgenesis. Our DNA constructs contain different temporally and spatially specific promoters, and hAβ42 linked to green fluorescent protein (GFP) by the viral 2A peptide. Importantly, Aβ42 is preceded by a zebrafish erythropoietin signal peptide (EpoSP) to ensure the cleavage and secretion of Aβ42, thereby mimicking the natural processing of Aβ42. The combination of highly efficient Tol2 transposon-based transgenesis, the ‘self-cleaving’ viral 2A peptide, and the numerous advantages of transparent zebrafish embryos provide a powerful technology by which the effects of hAβ42 can be continuously analyzed in living animals. We observed GFP expression in the injected embryos as early as 1 day post-fertilization. Immunohistochemistry of the injected embryos confirmed the expression of hAβ42 in and around GFP-positive cells, while acridine-orange staining of living embryos suggests increased cell death in proximity to the expressed hAβ42. Newly established transgenic lines with a heat-shock promoter and EpoSP-EGFP-V2A-hAβ42 are currently analyzed. Further confirmation of the validity of our zebrafish AD models, by detailed cellular and behavioral assays, is underway with an aim to develop these models for chemical modifier screening to identify potential drug candidates for AD treatment. Supported by NIH grant NS33981 and the North Carolina Biotechnology Center

920/B78
Prevalence of Brain-Reactive Autoantibodies in Human Sera and Their Cellular Targets in the Alzheimer's Disease Brain.
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Previous studies have reported immunoglobulin (Ig)-positive cells in post-mortem Alzheimer’s disease (AD) brains, which are only rarely observed in healthy, age-matched control brains. This
implies that brain-specific Igs exist in the sera of AD individuals and gain access to the brain interstitium. To investigate this possibility, we determined the prevalence of neuron-binding Igs in sera from AD patients, age-matched controls, and healthy younger individuals via immunohistochemistry and western blot analysis. Whole sera or its purified Ig fraction were applied as primary antibody to histological sections of human AD and age-matched control brains as well as adult mouse brains. Neuron-binding Igs were common in sera from AD and control individuals, with dramatic individual differences in the intensity and cell type specificity of neuronal immunolabeling. Interestingly, neurons in brain regions showing pathology were more reactive to serum Igs than the same neurons in comparable regions devoid of pathology. When sera or their Ig fractions were used to probe western blots of human, rat or mouse brain membrane proteins, a number of different protein targets were detected. Additionally, the relative number and intensity of bands in western blots tended to match the intensity of immunostaining of neurons in sections of human brain. Western analyses also suggested the presence of age- and disease-specific antibodies in some sera. Lastly, human serum Igs were found to immunolabel comparable neurons in histological sections of adult mouse brain cortical and hippocampal neurons and vice versa, suggesting cross-species reactivity. Results suggest a prevalence of neuron-binding Igs in human sera, a finding that leads us to propose that these Igs may be involved in the pathogenesis of AD and other neurodegenerative diseases that include BBB compromise. Supported by the National Institute of Aging (AG00925), the Alzheimer’s Association, and the New Jersey Governor’s Council on Autism.

921/B79
Neuron-Binding Autoantibodies in Human Sera Enhance Amyloid Beta\textsubscript{1-42} Accumulation in Adult Mouse Brain Neurons.
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A major feature of Alzheimer’s disease (AD) is widespread deposition of amyloid-beta peptides, especially amyloid-beta\textsubscript{1-42} (a\textbeta\textsubscript{42}), in neurons and amyloid plaques. Mechanisms for a\textbeta deposition in the brain and its association with aging remain to be elucidated. Previous studies have reported immunoglobulin (Ig)-positive neurons in post-mortem AD brains, suggesting that the blood-brain barrier (BBB) is permeable to Igs. We have observed a prevalence of immunopositive neurons in plaque- and neurofibrillary tangle-forming regions of AD brains that also exhibit substantial intraneuronal a\textbeta deposition. We hypothesize that chronic binding of Igs to neurons and Ig clearance via endocytosis enhance intraneuronal deposition of exogenous, soluble a\textbeta peptides in neurons. To investigate this, we examined the effects of human and mouse neuron-binding Igs on intraneuronal a\textbeta\textsubscript{42} deposition in adult mouse brain neurons In Vitro (organotypic brain slice cultures) and In Vivo (direct stereotoxic intracranial injection). Results revealed a dramatic enhancement in the rate and extent of exogenous a\textbeta\textsubscript{42} accumulation in mouse cortical and hippocampal neurons upon binding of human or mouse Igs to neurons. Moreover, Igs from individual sera that immunolabeled neurons most intensely in sections of human brain tissue were the most potent at enhancing intraneuronal accumulation of exogenous a\textbeta\textsubscript{42} peptide. Lastly, replacing whole serum or purified Ig fraction with purified antibodies directed against abundant neuronal surface proteins resulted in comparable enhancement of a\textbeta\textsubscript{42} accumulation in mouse brains both In Vivo and In vitro. Results suggest that neuron-binding Igs commonly present in human serum can gain access to neurons through defective BBB and enhance soluble a\textbeta\textsubscript{42} peptide deposition within neurons in AD brains via enhanced endocytosis. Therefore, brain reactive autoantibodies in human sera may be a significant risk factor for AD as well as other neurodegenerative diseases that have BBB compromise. Supported by the Alzheimer’s Association and the New Jersey Governor’s Council on Autism.

922/B80
Autophagic Regulation of Alpha-Synuclein Pathotoxicity Properties in Budding Yeast Reveals Unexpected Complexities.
Parkinson's disease (PD) is an incurable neurodegenerative disease characterized by the selective loss of dopaminergic neurons in the midbrain. This cell death is likely due to misfolding and aggregation of the protein alpha-synuclein. A prevalent hypothesis is that accelerating the degradation of alpha-synuclein can decrease cellular toxicity. Autophagy is a highly evolutionary conserved catabolic mechanism in eukaryotes used to recycle cell's own components such as damaged proteins. Pharmacological research implicates autophagy-based lysosomal degradation of alpha-synuclein, but genetic evidence is still lacking. We hypothesized that basal autophagy protects cells from alpha-synuclein toxicity and tested it in a budding yeast model in strains deleted for individual genes that control the three steps of autophagy: nucleation, expansion, or fusion. Thus far, we have examined four nucleation genes (Atg11, Atg17, Atg13, and Atg1), four expansion genes (Atg18, Atg4, Atg3, and Atg2), and two fusion genes (Vam3 and Vam7). We predicted accumulation of alpha-synuclein, its altered cellular localization, and increased cellular toxicity, in at least some of these strains. In fact, none of the gene deletions induced alpha-synuclein dependent cellular toxicity. However, alpha-synuclein localization showed subtle yet consistent changes when some Atg genes were absent. Specifically, in the absence of Atg11 and Atg2, alpha-synuclein aggregated and did not maintain plasma membrane localization. Without Atg17, alpha-synuclein became more cytoplasmically diffuse and less plasma membrane localized. To further our understanding of autophagy-mediated alpha-synuclein degradation, we will continue analyzing knockout strains for the remaining genes that compromise autophagy. (Supported by APDA, NSF-MRI, NSF-CCLI, & NIH R15)

923/B81

**Amyloid Beta Mediated Alterations to the Microtubule Associated Protein Tau.**

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Distinguishing hallmarks of Alzheimer's Disease include the accumulation of both amyloid beta (Aβ) plaques and neurofibrillary tangles (NFT's) in specific regions of affected brains. The aggregation of Aβ peptides into insoluble fibrils, the principal components of plaques, involves the production of Aβ soluble oligomer intermediates. NFT's are composed of insoluble aggregates of the microtubule associated protein tau. The amyloid cascade hypothesis suggests that Aβ soluble oligomers alter neuronal metabolism resulting in aberrant tau phosphorylation and truncation, ultimately leading to the production of NFT's and eventual neuronal death. In order to investigate specific effects of Aβ oligomers on tau, cultured rat hippocampal neurons were treated with oligomerized Aβ and a timecourse of biochemical samples were harvested prior to, and during, the period of cell death. Multiple phosphorylation epitopes on tau were analyzed for altered abundance via western blotting using site and phospho-specific antibodies. Surprisingly, none of the 8 phospho epitopes analyzed revealed altered levels within the timecourse of Aβ treatment relative to controls even though altered signaling pathways suggest elevated cdk5 and GSK3β activity within the neurons. on the other hand, a prominent alteration to tau observed throughout the timecourse of Aβ treatment was fragmentation, as seen by the loss of the full-length tau and accumulation of lower molecular weight fragments. These fragments remained stable in the later time points when significant levels of neuronal cell death was observed as assayed by both metabolic function and membrane integrity. Fragmentation of tau was preceded by increased calpain and caspase protease activity consistent with proteolytic action on tau. Taken together, these data indicate that an increase of phosphorylation at the sites we analyzed does not correlate with neuronal cell death whereas there is good temporal correlation between the generation of tau fragments and decreased neuronal viability. Overall, tau proteolysis may be a key functional step in Aβ mediated cell death.

924/B82

**Feed-Forward Mechanisms of Aβ Production and Secretion in Cortical Neurons.**
Sub-micromolar concentrations of dimeric/trimeric species of Aβ perturb synaptic plasticity in vitro and disrupt cognitive function in vivo. However, even in healthy individuals, Aβ is normally secreted but maintained at sub-toxic levels by various clearance mechanisms. Therefore, non-hereditary mechanisms that facilitate the sustained secretion of Aβ are of great interest for understanding the etiology of sporadic Alzheimer disease (AD). Based upon our findings that in neurons Aβ can induce cofilin-actin rods (rods), which block amyloid precursor protein (APP)-vesicle transport, we proposed a feed-forward mechanism by which neurodegenerative stimuli implicated in Alzheimer disease induce rods, block transport, and enhance Aβ secretion. Here, we report the development of a sandwich ELISA that quantitatively measures endogenous total rodent Aβ (Aβ1-40 + Aβ1-42) at levels down to 10 pg/ml. Using this ELISA and normalizing results to cell density by means of DNA assays, we demonstrate that levels of Aβ increase in response to treatments that generate rods, but only when neuronal ATP-levels remain high enough to promote the accumulation of APP-vesicles at rods. Peroxide (20 μM) treatment, which induces rods in 80% of the neurons and allows APP-vesicle accumulation, gives a 2.5 fold increase in secreted Aβ. Neither ATP depletion nor AMPA-treatment increases Aβ secretion or causes APP-vesicle accumulation, although both treatments induce abundant rods. No significant changes in intracellular Aβ occur under any treatment conditions. (Supported by NIH grant NS40371 to JRB).

925/B83
Glutamate Induced Cofilin/Actin Rods and Oxidation.
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Cofilin/actin rods are cigar-shaped actin aggregates that appear in neurites following a wide variety of stress that includes chemical ischemia, anoxia, cofilin over expression, or exposure to excess glutamate, peroxide, or amyloid beta 1-42 oligomers. Rod obstruction of neurite transport and synaptic function results in process degeneration distal to the rod. These rods are abundant in brain of Alzheimer disease (AD) patients but not in non-AD brain. To understand how stress involved in sporadic AD might generate the same pathology seen in familial AD, we are studying factors promoting rod generation. Herein we report that glutamate-induced rod formation likely requires inter-molecular cofilin disulfide bonds. Oxidized glutathione previously was shown in vitro to oligomerize cofilin through inter-molecular disulfide bonds generated between C39 and C147 by oxidation. Dimerized and oligomerized cofilin dramatically bundles actin into rod-like structures in vitro and has no disassembly activity. We adenovirally infected rat E18 hippocampal neurons to express a double cofilin mutant (CC39,147AA-mRFP) and exposed the neurons to excitotoxic levels of glutamate. Correlation coefficient analysis of neuronal confocal images showed that the double mutant had ~50% less tendency to incorporate into rods than wt cofilin-mRFP. In contrast to the double mutant, we found that single cofilin C/A mutants, which are still capable of inter-molecular disulfide bond formation, incorporate into rods as efficiently as wt cofilin-mRFP. It is important to note that the double mutant binds F-actin normally in sedimentation assays. Rods formed through cofilin over expression appear in unusually highly oxidized regions of neurites and are reversed by 15 min exposure to 4 μM β-mercaptoethanol.

926/B84
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The accumulation of amyloid beta (Aβ) peptides and formation of beta-amyloid plaques are central events in the development of Alzheimer’s disease (AD). Aβ is produced by the proteolysis
of amyloid precursor protein (APP). APP cleavage by different secretases and Aβ generation occur during APP intracellular trafficking. The Golgi apparatus, the central organelle for intracellular trafficking, however, has been observed fragmented in neurons of human AD patients. The molecular mechanism leading to Golgi fragmentation in AD and its effects on disease development remain elusive. In this study, we demonstrate that the Golgi is fragmented in the brain of APPswe/PS1dE9 double transgenic mice and in Chinese Hamster Ovary (CHO) cells stably transfected with APPswe/PS1dE9. Further experiments provided evidence that the Golgi fragmentation in AD is due to APP proteolysis and subsequent Aβ accumulation, which induces the phosphorylation of Golgi structural proteins and causes Golgi fragmentation. Expression of the N-terminal GRASP-Domain of the Golgi stacking factor GRASP65 rescued the Golgi structure and significantly reduced Aβ40 and especially Aβ42 production. Our results indicate that Golgi fragmentation in AD is caused by Aβ accumulation, which in turn affects APP trafficking and enhances Aβ production. Significantly, improving Golgi structure may alleviate the production of Aβ and decelerate the disease progress.

927/B85
Investigating the Contribution of Serine Phosphorylation, Alanine-76, and Familial Mutant E46k to A-Synuclein Pathotoxicity in Two Yeast Models.
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Parkinson disease (PD) is a devastating and incurable neurodegenerative disorder that affects over one million Americans. The universal PD pathology is the presence of aggregated α-synuclein within dying midbrain substantia nigra neurons. The role these α-synuclein aggregates play in cell death is unclear, and whether these aggregates are neuroprotective or harmful to cells is still being debated. Here, we first tested the hypothesis that serine phosphorylation promotes α-synuclein aggregation and toxicity by studying two phosphorylation-deficient (S87A & S129A) and two phosphorylation mimic (S87D & S129D) α-synuclein mutants in budding and fission yeast models. In budding yeast, we found that both S87A & S129A altered α-synuclein localization within cells and, surprisingly, increased cellular toxicity, but to differing extents in different strains. In fission yeast, both S87A & S129A redistributed α-synuclein within the cytoplasm, and increased toxicity to a mild extent. Secondly, we tested the hypothesis that alanine-76 (a key residue within the middle hydrophobic domain of α-synuclein) contributes to its aggregation and plasma membrane phospholipid association, by characterizing an A76E and A76R mutant in both yeasts. In support of this hypothesis, in budding yeast, significantly less A76E localized to the plasma membrane. Furthermore, in fission yeast, less A76E was aggregated in live cells. In both yeasts, A76E was found more cytoplasmically diffuse than wildtype α-synuclein. Finally, we examined the properties of E46K, the most recently discovered familial PD mutant. E46K bound to the plasma membrane in budding yeast and formed aggregates in fission yeast. Our data support the notion that serine-87 and serine-129 phosphorylation may make α-synuclein cytoprotective and alanine-76 aids in α-synuclein aggregation and membrane phospholipid binding. E46K had little influence on α-synuclein properties. E46K properties are more similar to wildtype α-synuclein and the A53T familial mutant than to the A30P familial mutant. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

928/B86
Mutant Huntingtin Mediates Neuronal Injury by Mitochondrial Fission.
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It is becoming increasingly clear that mitochondrial dysfunction is an early and central part of Huntington’s disease (HD) pathogenesis. The precise mechanism causing this defect is still
unknown. We therefore investigated whether mutant huntingtin (htt) alters the mitochondrial fission/fusion balance and thereby mediates neuronal injury and cell death. We find that mutant htt triggers polyQ length-dependent mitochondrial fission. EM tomography illustrates mitochondrial and cristae fragmentation in brain sections of mutant htt transgenic mice in vivo. Additionally, mutant htt interacts with the mitochondrial fission factor Dynamin related protein 1 (Drp1) in brain tissue of transgenic mice and post-mortem HD patients, and HD patient lymphoblasts in vivo. Furthermore, mutant htt accelerates Drp1 GTPase activity and modifies its oligomeric ring structure in vitro. Finally, mitochondrial fragmentation, arrest in anterograde and retrograde axonal transport of mitochondria, synaptic spine loss and neuronal cell death are all rescued by expression of Mitofusin 2 (Mfn2) and a dominant-negative Drp1 mutant. Thus, components of the mitochondrial fission and fusion machinery might be new therapeutic targets to combat neuronal injury in HD and perhaps other neurodegenerative disorders. Supported by: R01 NS047456, R01 EY016164, R01 NS055193, Hereditary Disease Foundation (to EBW) P41RR04050, R01 NS14718 (to MHE).

929/B87
Aberrant Membrane Trafficking from the Recycling Endosome in Huntington's Disease.
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Huntington's disease (HD) is a neurodegenerative disease caused by CAG repeat expansion (>37) in exon 1 of the gene encoding huntingtin. Whole brain atrophy and loss of neurons in the striatum and the cerebral cortex are hallmarks of HD pathology. There is no treatment to halt disease progression. Most studies to understand HD pathogenesis have focused on N-terminal fragments carrying the expanded polyglutamine tract. Analysis of the effects of the HD mutation on cell functions in the context of endogenous mutant huntingtin is more difficult because phenotypes are less robust and slower to develop than with the use of N-terminal fragments. However, detection and treatment of early phenotypes that precede onset of symptoms in HD patients may be valuable for slowing disease progression. Huntingtin associates with clathrin coated vesicles and interacts with proteins involved in vesicle budding and fusion. In this study, we used primary skin fibroblasts established from HD patients and primary cortical neurons of CAG140 knock-in HD mice as our models to investigate effects of mutant huntingtin on membrane trafficking in the endocytic pathway. We found that primary human HD fibroblasts and primary cortical neurons from homozygous CAG140 knock-in HD mouse embryos were normal in clathrin-dependent endocytosis of transferrin, but had deficits in post-endocytic recycling. Total Internal Fluorescence Reflection (TIRF) live cell imaging was used to track recycling vesicles with labeled transferrin. We found a redistribution of transport intermediates from small to large vesicles and from short to long tubules in primary HD fibroblasts, suggesting a deficit in vesicle budding at recycling endosomes. Biochemical assays showed that mutant huntingtin interfered with the nucleotide exchange on Rab11. Expression of dominant active Rab11 (Rab11Q70L) improved recycling of transferrin receptor in primary HD fibroblasts and increased survival of primary HD cortical neurons. Our study defines a novel mechanism for HD pathogenesis arising from defective Rab11-dependent membrane dynamics at the recycling endosome. Thus, approaches that increase Rab11 activity may be effective for treatment of HD.

930/B88
Aberrant Proteolysis of Polyglutamine-Expanded Androgen Receptor and Dynamics of Aggregation in SBMA.
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The aggregation of mutant and misfolded proteins is a common feature of many polyglutamine (polyQ) repeat disorders; the process by which these inclusions form is an important and unanswered question. Evidence from models of SBMA showing that nuclear inclusions (NI) lack C-terminal AR epitopes support the hypothesis that aberrant proteolysis of the expanded polyQ androgen receptor (AR) leads to accumulation of N-terminal AR fragments, a central process to SBMA pathogenesis. The protease and the circumstances of cleavage are currently unknown. In order to identify the AR proteolytic cleavage site, we immunoprecipitated AR aggregates from an inducible PC12 cell model exhibiting DHT- and polyQ length-dependent nuclear cleavage and aggregation of the expanded AR. Dissolution of NI with organic solvents resulted in the release of a 45 kDa N-terminal AR fragment that was analyzed by mass spectrometry for C-terminal residue identification. Preliminary results suggest that the cleavage point is at either amino acid D152 or L163. Precise identification and confirmation of the residue is ongoing. Studies are under way to identify the protease responsible for the aberrant AR cleavage; initial data suggest that the proteasome plays a role in AR aggregation. To examine the aberrant cleavage and aggregation event in SBMA, we created a PC12 cell line in which expanded AR is fused with CFP and YFP at the N- and C- termini, respectively. We noted that a proportion of cells contain AR aggregates that maintain the C-terminus, leading to the hypothesis that full-length AR is recruited to aggregates and subsequently cleaved. Stages of aggregation were classified based on aggregate size and C-terminus content, and live-cell fluorescent imaging was used to observe the aggregation process after DHT addition. From this we conclude that the aggregation stages represent a continuum of inclusion formation; moreover, that full-length AR aggregates prior to cleavage. A provisional rate of inclusion formation and maturation was determined and showed that inclusions form in 4-12 hours. These studies lay the groundwork for defining the role of AR proteolysis in SBMA pathogenesis as well as identifying the protease responsible for AR cleavage.

931/B89


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The disruption of the stoichiometry of neurofilament subunits (NFL, NFM and NFH for low, middle and high molecular weight neurofilaments respectively) can lead to the development of aggregates of these proteins, a hallmark of ALS. p190RhoGEF, a guanine nucleotide exchange factor found in mice, has been described that interacts with a small destabilizing region of NFL mRNA and imparts stability to the transcripts. p190RhoGEF is a critical determinant of murine NFL mRNA stability and alteration in its binding contributes to the formation of NF aggregates in mice. However, the function of this protein in humans, and its contribution to ALS pathology is unknown. Previously, we described the interaction of NFL mRNA with RGNEF, the human homologue of p190RhoGEF. This specific interaction was only observed in lysates from ALS affected spinal cords. In this study we have identified 4 distinct isoforms of RGNEF that we called A, B, C and D: the isoform a with 4956 bp; the isoform B with 5118 bp and an additional sequence in 3'; the isoform C with 5196 bp and an insertion before the additional sequence in 3' and the isoform D with 5061 bp similar to the isoform C but with a shorter RNA binding domain sequence than the others isoforms (917 against 1049 bp). We also show that at least two of these isoforms are expressed at mRNA level in the human nervous system with changes in the expression between control and ALS patient spinal cord tissues. These results suggest that the levels of the different RGNEF isoforms may play a role in the development of the ALS pathology, perhaps through distinctive patterns of interaction with NFL mRNA.

932/B90

Motor Neuron Degeneration Linked with Mitochondrial Fission in Amyotrophic Lateral Sclerosis.
Mitochondrial dysfunction is an early event in motor neuron degeneration during Amyotrophic Lateral Sclerosis (ALS). However, the precise cause of these mitochondrial deficits remains unclear. Mitochondria are dynamic organelles and cycles of mitochondrial fission and fusion ensure equal energy distribution across long axons, effective Ca2+ buffering, bio-energetic functionality, and translocation of the organelle to regions of high energy demands like the synapses. While mitochondrial fragmentation, or fission, contributes to neurodegenerative disorders and affects neurons with long axons such as motor neurons, its potential implication in ALS has never been explored. Therefore we tested whether mitochondrial fragmentation, or fission, by mutant SOD1 (mSOD1) contributes to motor neuron degeneration. To investigate this problem we co-cultured spinal cord motor neurons with astrocytes and used live cell imaging to track mitochondrial dynamics. Among the genes that cause familial ALS is sod1 which encodes Cu/Zn superoxide dismutase (SOD1). We find that mitochondrial length is decreased in motor neurons expressing mSOD1G93A suggesting that mitochondrial fragmentation may occur. In addition, axonal sprouting is decreased and correlates with an accumulation of small rounded mitochondria in the nerve terminal. Furthermore, both anterograde and retrograde transport of mitochondria is defective in mSOD1G93A motor neurons. Motor neurons expressing mSOD1G93A undergo rapid mitochondrial fragmentation in response to glutamate exposure and die faster. These data suggest that defects in mitochondrial dynamics may participate in ALS pathogenesis. Supported by: R01 NS047456, R01 EY016164, R01 NS055193 Timeliness: This study implicates for the first time mitochondrial fragmentation in ALS.

933/B91
The Effect of Ciprofloxacin on Toll-Like Receptor-4 Expression and Apoptosis Inducing Factor in Murine Microglial.
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Objective: to determine if AIF is modulated by a known therapeutic modulator of microglia activation. Mammalian Apoptosis-Inducing Factor (AIF) is a 67-kDa flavoprotein with a dual function of caspase independent cell death and a lesser studied mitochondrial oxidoreductase function in normal physiological conditions. Previous studies have shown that cells with reduced levels of AIF are resistant to PARP-1-dependent cell death. Other studies have linked AIF as a protein that can protect against oxidative stress via a type of antioxidant function. However, there are no known mechanistic strategies whereby the protective characteristics of this protein can be up-regulated without causing indirect cellular death. The aim of our study is to show that under LPS induced microglia stimulation a possible mechanism to reduce AIF’s translocation and possible enhance its protective mitochondrial oxidoreductase properties is through the use of a known toll-like receptor 4 inhibitor such as ciprofloxacin (CIP). Ciprofloxacin, a fluorinated 4-quinolone antibiotic, has modulatory actions as an anti-microbial and anti-inflammatory. Methods: Preliminary results were obtained by exposing lipopolysaccharides (LPS)-challenged BV2 murine microglial cells to various dose concentrations of ciprofloxacin. Samples were collected for analysis using standard western blot, immunocytochemistry, siRNA, In situ cell death detection, GSH depletion assay for AIF expression and translocation. Expression levels were determined for inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) for microglia activation. Results: LPS causes an increase in AIF, iNOS, and COX-2 expression levels in activated BV-2 cells. AIF expression and translocation correlated to a dose dependent response after exposure to 0, 10, 25, 50, 100mg/L concentrations of ciprofloxacin. No significant increase in cell death or changes in glutathione levels were detected. Conclusion: Ciprofloxacin, in a concentration-dependent manner, decreases AIF expression in activated BV-2 by attenuating the expression of toll-like receptor 4.

934/B92
Mysterious Growth and Locomotion of C. elegans.
The Huntington Associated Protein -1, HAP-1 (which is classified as T27A3.1 in C. elegans) is the first protein that is known to resemble the diseased protein Huntington. C. elegans is a model organism that can be used to study the binding protein T27A3.1. Because C. elegans contain the T27A3.1 protein in great abundance it was found to be useful when studying growth and locomotion of nematodes. An experiment was designed to determine if the T27A3.1 gene was knocked out of the nematodes, would the locomotion and growth be affected. The first assay was designed to study the movement of the nematodes. Four groups were used were used in this study. In one group T27A3.1 was present while in the other three groups, the gene was knocked out. The assay was performed by placing one L4 hermaphrodite on a NGM agar plate, which consisted of an E. coli bacteria lawn. The behavior of the nematodes was observed under a microscope for 3 minutes and the body bends of the worms were recorded and calculated. The same worms were then utilized for determining the effect that the gene had on the growth of the nematode. The worms remained on the plate at room temperature for 3 days and on the third day the number of offspring from the hermaphrodite was counted and recorded. The data collected proved that T27A3.1 had a significant effect on locomotion and growth. Data collected from the locomotion assay, indicated that the wild type nematodes did exhibit more body bends than the knock out nematodes. An ANOVA test proved a statistical difference between the groups containing the gene and the groups that were lacking the gene. In addition, the growth assay also showed a significant difference between those with the gene and those lacking the gene.

935/B93
Statins Differentiate Adult Rat Bone Marrow Stromal Cells into Neurons.
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PURPOSE: Neurons are obtained from differentiation of neural stem cells (NSCs). However, there are not so many NSCs in adult brains. Therefore, it is necessary to develop the method how to proliferate and differentiate endogenous NSCs or to find source of neurons other than NSCs. It has been known that bone marrow stromal cells (BMSC) are able to differentiate towards a neuronal phenotype. Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme a reductase, induce neurite-like outgrowth in PC12 cells and neurogenesis of NSCs after stroke. Therefore, the aim of the present study was to determine the effects of simvastatin, one of statins, on BMSC differentiation in vitro. METHODS: BMSC were collected from femurs and tibias of female Sprague-Dawley rats aged in 8 to 12 weeks. The cells were cultured in Dulbecco’s modified Eagle’s medium (low glucose). Subconfluent cultures of rat BMSC were plated to poly-D-lysine coated. Experiments were initiated by addition of 10 µM or 5 µM active form of simvastatin observing the effect on 24 hrs, 48 hrs, 72 hrs with Fluorescence-Activated Cell Sorting and RT-PCR. The expression of mRNAs encoding the neural markers NSE, β-tubulin III, NF68, and tau, encoding the neural markers, were determined by RT-PCR. RESULTS: BMSC isolated from the femurs of adult rats and propagated In Vitro were negative for CD11b, CD34 and CD45. The levels of expression of β-tubulin III, NF-68 and tau mRNAs were not detectable in uncommitted BMSC, however, were detectable after 24 hrs of neuronal induction. The levels of expression of NSE mRNA exhibited extremely low, however, incubation with the simvastatin for 24 hrs, the levels of expression of NSE mRNA greatly enhanced. CONCLUSION: These data indicate that simvastatin differentiated BMSC into neuron-like cells for 24 hours to be confirmed by the expression of mRNAs encoding the neural markers, NSE, β-tubulin III, NF68, and tau.

936/B94
Neuroprotective Effects of Cerium Oxide Nanoparticles Are Mediated by Scavenging of Nitric Oxide/Peroxynitrite.
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An increase in Nitric Oxide/Peroxynitrite (NO/ONOO-) in the nervous system triggers neuronal injury and cell death and plays a central role in the pathogenesis of acute and chronic neurodegenerative disorders. Thus alleviating NO/ONOO- stress may delay or even prevent neurodegeneration. Here, we explored the ability of cerium oxide nanoparticles (CeO$_2$ NPs) to scavenge NO/ONOO- in cell free systems In Vitro and in intact cortical neurons. Cerium oxide nanoparticles (CeO$_2$ NPs) are distinct from other rare earth metals in that they can alternate between the +3 and +4 state via redox/oxidation reactions at the particle surface and thus may act as autocatalytic antioxidants. Indeed we recently demonstrated that NPs with a high +3/+4 ratio are able to scavenge superoxide anions (O$_2^-$). Redox-cycling manganese-salen complexes that are effective Superoxide Dismutase (SOD) mimetics can also react with peroxynitrite (ONOO-). We therefore were wondering whether the CeO$_2$ NPs similar to SOD mimetics may scavenge NO/ONOO-. Here, we provide evidence that CeO$_2$ NPs interact with ONOO- in vitro. Interestingly, CeO$_2$ NPs provided neuroprotection of cortical neurons exposed to an exogenous NO donor. To test further whether the NP would also be able to scavenge intracellular NO/ONOO- we exposed neurons to toxic levels of glutamate. Glutamate activates the neuronal glutamate receptors and triggers a rapid rise in Ca$^{2+}$, which in turn activates Nitric Oxide Synthase, leading to NO/ONOO- production. Interestingly, the NP provided significant neuroprotection against glutamate-induced excitotoxicity. Furthermore, we show that the CeO$_2$ NPs indeed entered the neurons and were often found in association with mitochondria using EM microscopy. Since CeO$_2$ NPs react with ONOO- in vitro, these results point to an In Vivo scavenging activity of ONOO- by CeO$_2$ NPs. Thus, the radical scavenging properties of CeO$_2$ NPs may offer new opportunities to combat neurodegenerative disorders. Supported by NIH grants R01NS047456, R01 EY016164, R01 NS055193 (to EBW). NIH NCRR grant P21 RR04050, ES010337. DK 54441 (to MHE). NIH R01AG031529-01 (to WTS and SS), as well as NSF NIRT N11RT CBET 0708172 (to SS and WTS).

937/B95
Effects of Lysosomal-Mediated Regulation of Autophagy Pathway on Neuronal Mitophagy.
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Autophagy is a lysosome-dependent cellular degradation and homeostasis pathway, which, along with proper mitochondria quality control and turnover, has been implicated in the pathologic processes of neurodegenerative diseases. In non-neuronal cells, mitophagy has been shown to be responsible for mitochondrial turnover (Narendra et al., 2008), however, significance of macroautophagy in neuronal mitochondria quality control is not yet well characterized. Here, using both cellular and biochemical approaches on cultured neurons, we establish that proper lysosomal function is necessary for autophagosome clearance in neurons and implicate a critical role of this pathway in mitochondria quality control. First, we observe autophagy upregulation after application of lysosome enzyme inhibitors in neurons. Second, we show that mitochondria turnover is mediated by macroautophagy. Third, through the use of CCCP, a mitochondria decoupler, we demonstrate that damaged or depolarized mitochondria are specifically targeted through the autophagy pathway for clearance. Because impaired autophagy and mitochondria dysfunction have been implicated in a number of neurodegenerative diseases, our study provides a cellular clue for further investigation into the relative contribution of the autophagy pathway to pathology or pathogenesis of relevant neurodegenerative mouse models, such as those for ALS and Alzheimer’s Disease. (Supported by the Intramural Research Program of NINDS, NIH)

938/B96
Contribution of the NR2B Subunit of the NMDA Receptor to Neuronal Calpain and Caspase-3 Activation Following Mechanical Injury In Vitro.
Neuronal cell death following traumatic brain injury (TBI) is associated with the stimulation of the N-methyl-D-aspartate receptor (NMDAR), leading to subsequent activation of calpains and caspases. In the present study, the effect of NMDAR antagonists on protease activation following excitotoxic or mechanical injury In Vitro was evaluated. Cortical neurons at 18 days In Vitro were treated with either vehicle (salt solution), the competitive NMDAR antagonist APV, or the non-competitive, NR2B subunit-selective antagonists (ifenprodil or Ro-25,6981) and were then subjected to either excitotoxic (NMDA) or mechanical (stretch) injury. Neurons exhibited protease activation between 30min and 24h following exposure to 100μM NMDA with caspase-3 activation predominating at 30min and calpain activation predominating at 24h. Both ifenprodil and Ro-25,6981 (1-100μM) significantly reduced caspase-3 activation at 30min (p<0.05) while APV (25μM) was effective at 4h (p<0.05). Calpain activation was inhibited by APV (10-25μM) at 4h (p<0.001) and 24h (p<0.001) and ifenprodil (10μM) at 30min (p<0.05) and 24h (p<0.005), whereas Ro-25,6981 had no effect. Interestingly, ifenprodil at 100μM exacerbated activation of both caspase-3 and calpain at 24h (p<0.001). Following either moderate or severe stretch injury, activation of both calpain and caspase-3 was observed at 30min and 4h but not at 24h. In contrast, mild stretch only activated caspase-3 at 24h. Moderate, but not severe, stretch injury in the presence of either competitive or non-competitive NMDAR antagonists (10μM) significantly inhibited calpain activation (p<0.05) as compared to vehicle treatment. Caspase-3 activation post stretch was not affected. Collectively, these data suggest that NMDARs, including NR2B-containing NMDARs, may mediate calpain activation following mechanical trauma, albeit in a severity-dependent manner. Conversely, caspase-3 activation following stretch injury appears to be independent of NMDAR stimulation. These findings underscore the complexities of neuronal responses to traumatic brain injury.

939/B97
Expression of PTEN Induced Putative Kinase 1 (Pink1) Alters the Effects of Foxo Overexpression in Drosophila.
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PTEN induced putative kinase 1 (PINK1) has been shown to play a key role in preventing mitochondrial dysfunction during cellular stress. PINK1 has been demonstrated to act upstream of parkin and interact with several mitochondrial proteins, but its involvement with the insulin receptor (INR) pathway has been largely overlooked. Clues to this interaction include its activation through the dual role of PTEN, an indirect interaction with AKT through parkin, and transactivation by Forkhead box O (Foxo) transcription factors. To further investigate the influence of Pink1 on the INR pathway, we examined the directed expression of Pink1, Pink1 mutants, and Pink1 interfering RNAs (RNAi) with the overexpression of Foxo in the developing eye of Drosophila. Overexpression of Foxo in the Drosophila eye leads to a characteristic phenotype of reductions in cell size and cell number, and can be rescued by co-expression of upstream insulin signaling components. As a protective protein, we looked at the ability of Pink1 to rescue this phenotype. Interestingly, co-overexpression of Foxo and Pink1 showed an increase in the severity of the phenotype, with decreased cell size and number. A partial rescue was observed only with the Pink1 RNAi, suggesting that Pink1 is able to increase the pro-apoptotic effects of Foxo. This is contrary to the view that PINK1 acts exclusively as a protective protein in the cell. It is likely that PINK1 is involved in aspects of cell fate decisions other than mitochondrial protection. Support contributed by: Memorial University School of Graduate Studies Fellowship to A.M. Todd, Parkinson Society Canada Friedman Pilot Project Grant and NSERC Discovery Grant to B.E. Staveley.
Other Diseases II (940 -959)

940/B98
Fluid Shear Stress Modulates Macrophage-Induced Urokinase Plasminogen Activator Expression in Osteoarthritic Chondrocytes.
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Mechanical stress initiates the cartilage lesion by altering chondrocyte-matrix interactions and inducing synthesis of matrix-degrading proteinases by chondrocytes. In addition, macrophage-like cells of the synovium release proinflammatory factors which are responsible for the upregulation of cartilage breakdown proteinases in chondrocytes, and play a critical role in cartilage degradation during osteoarthritis (OA). However, the interplays among macrophages, chondrocytes and shear stress in regulating cartilage function are not clearly understood. In this study, we investigated the mechanisms underlying the modulations of human OA chondrocyte uPA expression by macrophages and shear stress. Stimulation of OA chondrocytes with macrophage-conditioned medium induced time-dependent uPA expression and phosphorylation of ERK, JNK, p38, and Akt. By using specific inhibitors, we demonstrated that activation of JNK and Akt pathways are critical for the macrophage-induced uPA expression. Transcription factor ELISA assay showed that macrophage-conditioned medium increased nuclear factor (NF)-κB-DNA binding activity in OA chondrocytes. Inhibition of NF-κB activation by specific inhibitor blocked the macrophage-induced uPA promoter activity and expression. In addition, Shear stress was applied to human OA chondrocytes following the stimulation of macrophage-conditioned medium. Shearing of OA chondrocytes at 5 and 10 dyn/cm2 inhibited the macrophage-induced JNK, Akt phosphorylation, NF-κB activation, and uPA expression. Our findings serve to elucidate the molecular mechanisms underlying the macrophage induction of uPA expression in human OA chondrocytes and the shear stress protection against this induction.

941/B99
Homocysteine Induces Cell Proliferation through Up-Regulation of Cyclin D1 Expression via B1 Integrin/Akt/P70s6k Signaling Pathway in Human Vascular Smooth Muscle Cells.
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The molecular basis of homocysteine-induced cell proliferation in human vascular smooth muscle cells (SMCs) remains unclear. We investigated the mechanism by which homocysteine affects the cyclin D1 expression in human umbilical artery smooth muscle cells (HUASMCs). Homocysteine treatment for 24 and 48 h induced proliferation of cells. Homocysteine increased the protein level and promoter activity of cyclin D1. We further examined the mechanism for the regulation of cyclin D1 expression and cell proliferation. Akt and p70S6K signaling pathway was investigated on the responses of cyclin D1 to homocysteine. Homocysteine induced the transient phosphorylation of Akt and p70S6K, and treatment with phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) and mammalian target of rapamycin (mTOR) inhibitor (rapamycin) attenuated the homocysteine-induced up-regulation of cyclin D1 expression and cell proliferation. The integrin antagonist RGD peptide and β1 integrin small interfering RNA (siRNA) blocked cell proliferation and cyclin D1 expression as well as Akt and p70S6K phosphorylation. This homocysteine-induced β1 integrin/Akt/p70S6K signaling pathway and expression of cyclin D1 may accelerate progression of atherosclerotic lesions by promoting proliferation of vascular smooth muscle cells.

942/B100
The Effect of Bevacizumab and Other Growth Factors on the Damage of Corneal Cells.
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Purpose: to evaluate apoptosis induced by bevacizumab and protective effect of other growth factors on bevacizumab induced cellular damage in corneal cells Methods: to evaluate induction of apoptosis in corneal epithelial cells and fibroblast by application of bevacizumab, LDH, FACS analysis, annexin V stain, and analysis of expression of apoptosis related markers such as bax, bcl-2, and bcl-xl were done. To reveal the mechanism of bevacizumab induced cellular toxicity and to suggest regulator of the toxicity, after co- treatment of bevacizumab and fetal bovine serum (FBS), EGF, FGF, or TGF-β to epithelial cells and fibroblast, LDH, FACS analysis, annexin V stain, analysis of expression of apoptosis related markers repeated. Results: The apoptotic change of corneal epithelial cells and fibroblasts cells were increased after application of bevacizumab with concentration dependent manner. The expression of bax, bcl-2, and bcl-xl also changed compatible to apoptotic condition. After co- treatment of bevacizumab and FBS or EGF, the level of LDH, Annexin V stain, and FACS analysis showed their inhibitory affect on apoptotic change and cellular damage. Conclusions: Bevacizumab has not only vascular suppression effect but induces apoptosis and cellular damage in corneal epithelial cells and fibroblasts. This toxicity of bevacizumab was inhibited by application of FBS or other known growths factors.

943/B101
Human Lactoferrin Down-Regulates Tnf-α-Induced Intercellular Adhesion Molecule-1 Gene Expression via NF-κB Inhibition in Endothelial Cells.
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Intercellular adhesion molecule-1 (ICAM-1) is up-regulated on numerous cell types in response to a variety of inflammatory mediators. TNF-α stimulates the ICAM-1 gene transcription mainly via a variant NF-κB site located from -187 to -178 bp upstream of the transcription start site. In the present study, we provide biochemical and functional evidence that lactoferrin (Lf), a DNA binding protein, down-regulates TNF-α-induced ICAM-1 gene expression via inhibiting translocation and binding of NF-κB. We demonstrated that Lf reduced TNF-α-induced mRNA and protein levels of ICAM-1 in endothelial cells. ICAM-1 promoter reporter assay indicated that Lf downregulates ICAM-1 at the transcriptional level. TNF-α-induced DNA binding of NF-κB to the variant NF-κB site was significantly inhibited by treatment of Lf. A putative Lf binding site was found to in the ICAM-1 promoter which is overlapped with the NF-κB site. It was demonstrated that exogenous Lf was transported into nucleus in endothelial cells and that Lf alone can bind to the LBS/NF-κB site. ABCD assay demonstrated that Lf competed with NF-κB for the binding to the site. Lf also inhibited TNF-α-induced phosphorylation of IkBα and nuclear translocation of the p65 subunit of NFκB. Furthermore, Lf inhibited U937 monocytoid cell adhesion to HUVECs stimulated with TNF-α. Our results demonstrate that Lf inhibits expression of ICAM-1 via reducing nuclear translocation of NFκB as well as interfering NF-κB binding to the variant NF-κB site, suggesting a protective role of Lf for vascular inflammatory diseases.

944/B102
Human Plasminogen Kringle 1-5 Reduces Atherosclerosis and Neointima Formation in Mice by Suppressing the Inflammatory Signaling Pathway.
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Background: Activation of vascular endothelial cells (ECs) plays an important role in atherogenesis and plaque instability. Recent research demonstrated that late-stage inhibition of plaque angiogenesis by angiostatin (kringle 1-4) reduces macrophage accumulation and slows the progression of advanced atherosclerosis. Kringle 1-5 (K1-5) is a variant of angiostatin that contains the first five kringle domains of plasminogen. Objective: to investigate whether K1-5 has an inhibitory effect on early-stage atherosclerosis using the apolipoprotein E (ApoE)-deficient mice model and a carotid artery ligation model. Methods: ApoE-deficient mice received K1-5 treatment for 4 weeks, and the severity of aortic atherosclerosis was measured. In the ligation
model, the left common carotid arteries of C57BL/6 mice were ligated near the carotid bifurcation, and the mice received K1-5 for 4 weeks. Human umbilical vein endothelial cells were pretreated with K1-5 before TNF-α treatment to explore the anti-inflammatory effect of K1-5. Results: The areas of the lesion in the aortas of ApoE-deficient mice that received K1-5 treatment were notably decreased, and formation of carotid neointima in the C57BL/6 mice was decreased by treatment with K1-5. Expression of TNF-α-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 was inhibited by K1-5 treatment, possibly via down-regulation of translocation of nuclear factor-κB and expression of reactive oxygen species. Conclusions: K1-5 reduced atherosclerosis and neointima formation in mice, possibly through inhibition of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in ECs.

945/B103

**Hepatocyte Hedgehog Pathway Activity in Liver Injury.**

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Background and Aims: We have previously identified Hedgehog (Hh) pathway gene expression in human cirrhotic liver. Upregulated Hh pathway activity is also associated with hepatocellular carcinoma development. However, the functional significance is unknown. Therefore, this study aimed to characterise the role of the Hh pathway in an In Vivo mouse model of progressive liver injury. Methods: C57BL/6 mice (n = 5-10) were administered thioacetamide (TAA 300mg/L; drinking water ad lib) for 4, 8 or 20 wks. Hh component expression in whole liver and primary hepatocyte samples was determined using in situ hybridisation (ISH), immunohistochemistry (IHC), reverse-transcriptase PCR (RT-PCR) and quantitative real-time PCR (qPCR). Results: TAA induced a time-dependent progression of liver injury resulting in end stage cirrhosis. Whole liver RT-PCR confirmed expression of Hh components in control and treated groups. qPCR analyses confirmed significant upregulation of Hh pathway activity with progressive liver injury, indicated by upregulation of Gli1 (transcriptional activator) and downregulation of Hhip (Hh inhibitor). RT-PCR analyses also confirmed expression of Hh pathway components in untreated primary hepatocytes. Gli1 expression was induced in primary hepatocytes with progressive liver injury, indicative of Hh pathway activation. In normal liver, ISH revealed specific localisation of Shh (ligand) to cells in the portal tract and diffuse expression across the liver lobule. Expansion of Shh expressing cell populations occurred with TAA-induced liver injury, within the fibrous septae. Shh localised to hepatic stellate cells, immune cells, oval cells, vascular endothelial cells and hepatocytes (IHC). Conclusion: Our studies demonstrate that the Hh signalling pathway is active in normal liver and is upregulated with progressive liver injury. Injured hepatocytes were found to have active Hh pathway signalling, with activity upregulated with progressive liver injury. Hepatocytes were also shown to produce Hh ligand. These results indicate that the hepatocyte may contribute to intrahepatic intercellular interactions involved in progressive liver injury, through Hh mediated paracrine or autocrine mechanisms.

946/B104

**Preparation of a Controllable RNA Polymerase I-Dependent Expression Vector.**

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Objective: Twenty-million people die from infection diseases in the world a year. Most infectious diseases are caused by RNA virus. The RNA genome transfer system is a powerful tool for basic and clinical research of infection. Mammalian cells have three types of RNA polymerase (pol): RNA pol I, II and III. RNA pol I, II and III transcribe ribosomal RNA, most genes encoding proteins
and various small RNAs, respectively. A variety of non-viral and viral vector systems has been developed with RNA pol II and III promoters; however, vectors with RNA pol I promoters have never been fully developed. We previously prepared RNA pol I-dependent plasmid vector. In the present study, we developed tetracycline (tet)-controllable plasmid and adenovirus (Ad) vectors with RNA pol I promoter. Methods: RNA pol I promoter is composed of enhancer, upstream control element (UCE) and core promoter. To optimize tet-controlled RNA pol I cassette, tet operator (tetO) sequence was inserted into the various sites of RNA pol I promoter. Luciferase was used as a reporter gene. Ad vectors were constructed by an improved In Vitro ligation method (Mizuguchi and Kay, 1998, 1999). Results: TetO sequence was inserted upstream of enhancer, core promoter or UCE in RNA pol I promoter sequence. Co-transfection of the tet-regulated RNA pol I plasmid with plasmid containing a tetracycline repressor or activator resulted in dose-dependent decrease or increase in luciferase expression, respectively. Ad vectors containing the tet-regulated RNA pol I cassette exhibited tet-controllable luciferase expression. We also applied this vector system into evaluation of replication of hepatitis C virus (HCV), and we developed a novel HCV replication system using RNA pol I system. Conclusion: We developed plasmid and viral vector with a tet-controllable RNA pol I promoter. This system will contribute to overcoming infection of RNA viruses.

947/B105
β3 Integrin Is Involved in Cardiac Fibroblast Proliferation, Pyk2 Signaling and Extracellular Matrix Production.
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Our recent studies indicate that β3 integrin contributes to hypertrophic growth and survival of cardiomyocytes in response to pressure-overload induced by transverse aortic constriction (TAC). In addition to differences in cardiomyocyte survival after TAC, β3 integrin knockout (β3 KO) animals exhibited reductions in collagen content, fibronectin (FN) levels, and Pyk2 phosphorylation in comparison to wild-type (WT) mice. As collagen and FN are produced primarily by cardiac fibroblasts (Cfb), we sought to assess the relevance of β3 integrin in Cfb in hypertrophic myocardium. Hence, we compared adhesion and platelet-derived growth factor (PDGF-BB) signal transduction, a growth factor with increased expression in hypertrophic myocardium, in isolated primary Cfb from WT and β3 KO mice. In comparison to WT cells, the number of β3 KO Cfb adhered to FN and VN was decreased at 30 min after plating. Similarly, spreading in the initial period (< 30 min after plating) was reduced in β3 KO Cfb when compared to WT Cfb. These data indicate that β3 integrin might play a crucial role in the initial attachment and spreading of Cfb. To understand signaling pathways downstream of β3 integrin, we analyzed the phosphorylation state of nonreceptor tyrosine kinases, the classical components of adhesion signaling. While the phosphorylation of Src and FAK in β3 KO Cfb upon adhesion to FN was similar to that of WT Cfb, there was a marked reduction in Pyk2 (Y402) phosphorylation in β3 KO Cfb. In addition, upon PDGF-BB treatment, while there was no difference in Src and FAK phosphorylation, Pyk2 phosphorylation was markedly reduced. These data suggest that β3 integrin influences Pyk2 signaling in Cfb upon adhesion and growth factor stimulation. Since integrin-Pyk2 signaling is involved in cell proliferation and ECM production, we next analyzed for these changes in WT and β3 KO Cfb. Our results indicate a significant reduction in PDGF-BB-stimulated cell proliferation and collagen production in β3 KO Cfb when compared to WT cells. Together our study demonstrates that, in addition to effects on cardiomyocytes, β3 integrin influences the proliferation and function of Cfb possibly via Pyk2 to affect ECM remodeling in cardiac hypertrophy.

948/B106
Cytoprotective Effect of P38β MAPK in Astrocytes under Oxidative Stress.
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Four distinct genes encode the four known members of p38 mitogen-activated protein kinases (MAPKs), that is, p38α, p38β, p38γ, and p38δ in mammals. The activation of p38 MAPKs has been implicated in many cellular processes, such as, inflammation, cell death and survival. Despite the fact that p38α and p38β MAPKs share over 75% homology sequences, increasing evidence indicates that they have distinct, perhaps even opposite roles under stress conditions. We have shown that p38β MAPKs was induced in activated astrocytes, which are enriched in the penumbra after transient focal cerebral ischemia. To investigate the functional significance of p38β MAPK in astrocytes, a C6 astrogloma cell line stably over-expressing p38β MAPK was generated. In these cells, hydrogen peroxide-induced apoptosis was 60% of that compared to parent cells. Interestingly, we found that expression of the small heat shock proteins, αB-crystallin, was significantly increased in these cells, but that the expressions of HSP27 and HSP70 were not. Furthermore repression of αB-crystallin expression by αB-crystallin siRNA transfection suppressed this protective effect indicating that αB-crystallin induction has a crucial role in the protection against H2O2-induced apoptosis in p38β-overexpressing C6 astrogloma cells. In addition, the induction of caspase 3 activity after H2O2-treatment was markedly suppressed in p38β-overexpressing cells, and immunoprecipitation proved binding between αB-crystallin and partially processed caspase-3. These results indicate that p38β confers protection against H2O2-induced astrocytes apoptosis by inducing the small heat shock protein, αB-crystallin, which inhibits caspase-3 activation.

949/B107
Sophora Flavescens Aiton Inhibits the Production of Pro-Inflammatory Cytokines through Inhibition of the NFκB/IkB Signal Pathway in Human Mast Cell Line (Hmc-1).
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Objective: The dried roots of Sophora flavescens Aiton (SFA) has been used in traditional medicine for treatment of inflammation, gastrointestinal hemorrhage, diarrhea, and asthma. This study investigated the effect of SFA on the inflammatory allergic reaction. Methods and Results: SFA (200 mg/kg) inhibited the mast cell-mediated passive cutaneous anaphylaxis reaction in vivo and the release of histamine from rat peritoneal mast cells by compound 48/80. In addition, the expression levels of phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187-stimulated TNF-α, IL-6, and IL-8 were also decreased by SFA treatment. In molecular mechanism level, this study showed that SFA inhibited the nuclear translocation of nuclear factor (NF) κB through inhibition of the phosphorylation and degradation of IκB-α, which is an inhibitor of NF-κB. Moreover, SFA suppressed PMA plus A23187-induced phosphorylation of the mitogen-activated protein kinase p38 and c-jun N-terminal kinase. The inhibited induction of NF-κB promoter by SFA was determined using luciferase activity. Conclusions: These results suggest that SFA could be used as a treatment for mast cell-derived allergic inflammatory diseases.

950/B108
Functional Studies of Primary Hepatocytes: The Relationship between CD147 Expression and Matrix Metalloproteinase (MMP) Activity.
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Introduction: CD147, a potent inducer of MMPs (1, 2, 3, 9 & 14) is upregulated in human cirrhotic liver and expressed predominantly on hepatocytes. Additionally, CD147 is the only known receptor for pro-inflammatory extracellular cyclophilin a (CyPA). Progression of liver fibrosis is
characterised by the dysregulation of extracellular matrix (ECM) turnover, mediated by MMPs. Therefore, this study examined expression of CD147 and MMPs in the primary hepatocyte, to determine if the hepatocyte has a role in ECM remodeling. Methods: Cellular localization of CD147 and MMPs (1, 2 & 9) was determined by IHC and confocal microscopy of human cirrhotic liver explants. In-vitro studies were performed on isolated primary mouse hepatocytes from C57Bl6 mice. Cells were cultured on plastic, collagen-1 or matrigel and treated with both CD147 binding [anti-CD147 antibody (5ug/ml) and CyPA (10nM)] and non-binding (TNF-α, TGF-β, HGF) stimuli for 24 hours. An anti-Fc antibody was used to determine the MMP induction specificity of CD147 ligands. CD147 expression was analysed by Western blotting and MMP 2 & 9 activity was determined by gelatin zymography. Results: Localization studies demonstrated that CD147 was expressed on cytokeratin-18 positive hepatocytes as well CD31 positive endothelial cells and CD45 positive inflammatory cells. Further, IHC demonstrated that MMP-1, 2 & 9 were expressed on hepatocytes. In-vitro functional studies of primary hepatocytes demonstrated that CD147 protein expression was most pronounced on matrigel and was induced by anti-CD147 antibody as well as TNF-α, TGF-β, and HGF. Further, MMP-9 expression was also induced by TNF-α. Moreover, either anti-CD147 or IgG control antibody when combined with CyPA, upregulated MMP-2 and -9 dramatically. This induction was blocked by pre-incubation of hepatocytes with an anti-Fc receptor blocking antibody, however anti-CD147 administration was still able to induce MMP-9. Conclusion: The observed upregulation of CD147 and MMP-9 expression in-vitro, suggest that hepatocytes have inducible MMP expression. MMP induction with anti-CD147 administration is consistent with hepatocyte-derived CD147 being actively involved in ECM remodeling associated with liver fibrosis.

951/B109
Toxicity of Isoproterenol Increases with the Differentiation State of H9c2 Myoblasts.
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Cardiomyocyte apoptosis is one consequence of several cardiovascular diseases. During heart failure, larger amounts of catecholamines are released to induce an attempt to restore homeostasis. Over-activation of beta-adrenergic receptors (AR) leads to cardiomyocyte death. Rat H9c2 myoblasts are usually used as an In Vitro model for cardiac cells and are normally used in its undifferentiated state. To investigate the relevance of the differentiation state on drug toxicity, we used three different experimental groups: undifferentiated H9c2 myoblasts, H9c2 differentiated to myotubes in a low serum medium and H9c2 differentiated to a cardiac phenotype by incubation in a low serum media with daily additions of retinoic acid (RA). The objectives of the present work were to characterize the susceptibility of H9c2 myoblasts in different differentiation states to the beta-adrenergic agonist isoproterenol (ISO) and correlate drug effects in terms of alterations in pro-apoptotic proteins and beta-adrenergic receptor density. We observed that by manipulating the differentiation state of H9c2, a different response of cells to ISO was obtained, where differentiated H9c2 cells being more susceptible than non-differentiated cells to ISO for 24 and 48 hours of exposure. Interestingly, differentiated H9c2 cells with RA appear to have a lower amount of beta2-AR (involved in the anti-apoptotic pathway) and beta3-AR than the other two groups of cells. Both the mitochondrial voltage-dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT) were analyzed as both proteins are part of the mitochondrial permeability transition pore. Although no differences regarding the ANT were observed, differentiated cells in the presence of RA contained lower amounts of VDAC. No other differences between groups were found regarding the apoptosis inducing factor (AIF), Bax and Bcl-2, proteins involved in the apoptotic signaling pathway. The present study suggests that the differentiation of H9c2 myoblasts is relevant for the toxicity of isoproterenol, although the molecular mechanism is still under investigation. This work is supported by Fundação para Ciência e Tecnologia, Portugal (FCT PTDC-QUI-64358-2006, SFRH BD/41384/2007).
952/B110
Bone Morphogenetic Protein Recruits a Peroxisome Proliferator-Activated Receptor Gamma and Beta-Catenin Complex to Regulate Apelin Expression in Pulmonary Endothelial Cells.
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In patients with pulmonary arterial hypertension (PAH), mutations in bone morphogenetic protein receptor (BMPR)II signaling result in PA endothelial cell (EC) dysfunction causing reduced EC survival and heightened smooth muscle cell (SMC) proliferation. The down-stream gene targets of BMP signaling important in the pathogenesis of PAH are poorly characterized. Interventions that normalize expression of genes that are targets of BMPRII-mediated signaling could restore PAEC function and reverse PAH. Our data shows that in PAEC BMP signaling can promote physical interaction between nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) and beta-catenin (BC), and disruption of this complex with synthetic PPARγ ligands impairs BMP2 mediated survival response. We hypothesized that BMP induced PPAR-BC transcription factor complex regulates genes necessary for PAEC homeostasis and important in PAH. Using whole genome wide ChIP-Chip and gene-expression microarray techniques we have characterized novel targets for BMP-PPAR-BC signaling axis, and identified apelin as an important novel target. The significance of our finding in PAH is shown with BMPRII deficient pulmonary EC from idiopathic PAH (IPAH) patients expressing significantly lower levels of apelin than those from control patients. We further show that deletion of PPARγ from EC in mice, previously shown to cause PAH phenotype, is associated with a significant reduction in apelin expression. Administration of apelin for 2 weeks significantly reverses PAH in these PPARγ deficient mice. Our In Vitro experiments demonstrate that apelin-deficient PAEC are prone to apoptosis and promote PASMC proliferation. We conclude that decreased apelin expression is associated with dysfunctional BMP signaling and contributes to pulmonary vascular remodeling. We further show evidence that apelin could have therapeutic implications in PAH.

953/B111
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The βA3/A1-crystallin gene, which codes for a major structural protein of the lens, is mutated in the Nuc1 rat. We have recently shown that βA3/A1-crystallin is also expressed in astrocytes; however, its function in astrocytes remains elusive. Our studies with Nuc1 suggest that βA3/A1-crystallin may have an important role in maintaining the nuclear architecture of astrocytes, where βA3/A1-crystallin is co-localized with the nuclear membrane protein, Emerin. The mutation causes nuclear membrane fragility, change in nuclear shape and altered disposition of chromatin. In Nuc1 astrocytes, the nucleolus is larger, singular and consistently located at the periphery of the nucleus rather than in the more central location typical of normal astrocytes. In addition, unique perinuclear vacuolar structures containing amorphous and electron-dense material are present in the mutant astrocytes. We suspect that these structures are autophagic in nature. The activation of autophagy is supported by an increase in Beclin 1 in Nuc1 astrocytes compared to normal during early postnatal development. Beclin 1 has been postulated to be important for the initiation of the formation of the autophagosome. This may suggest that autophagy is involved in the rapid repair of the damaged nuclear components of the Nuc1 astrocytes. The autophagic clearance of nuclear wastes in Nuc1 astrocytes appears to diminish as excess degradation of the nuclear components becomes more profound in the aging Nuc1 rats. Indeed, our immunolabeling studies clearly show that Beclin 1 is downregulated in the astrocytes of aging Nuc1 rats. A failure of the programmed cell death process, as we have reported in Nuc1 during ocular development, followed by the failure of autophagy in astrocytes would lead to necrosis and may be the basis for retinal degeneration in aging Nuc1 rats.
954/B112
Structural and Physiological Studies of Filamin A-CFTR Interaction.
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Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane chloride channel expressed in epithelial cells. A previous report has shown that filaminA (FLNa), an actin cross-linking and scaffolding protein, directly interacts with the cytoplasmic N-terminus of CFTR and that this interaction is necessary for stability and confinement of the channel. Here we report that the CFTR N-terminus has sequence similarity to known FLNa-binding partner binding sites. FLNa has 24 Ig (IgFLNa) repeats. A CFTR peptide pulled down repeats 9, 12, 17, 19, 21, and 23, which share sequence similarity but differ from the other FLNa Ig domains defective in CFTR binding. Using known structures of IgFLNa-partner complexes as templates, we generated in silico models of IgFLNa-CFTR peptide complexes. Point and deletion mutants of IgFLNa and CFTR informed by the models, including disease-causing mutations, L15P and W19C, disrupted the binding interaction. The model predicted that a P5L CFTR mutation should not affect binding, but a synthetic P5L mutant peptide had reduced solubility, suggesting a different disease-causing mechanism. Taken together with the fact that FLNa dimers are elongated (~160 nm) strands, whereas CFTR is compact (6~8 nm), we propose that a single FLNa molecule can scaffold multiple CFTR partners. Unlike previously defined dimeric FLNa-partner complexes, the FLNa-monomeric CFTR interaction is relatively weak, presumptively facilitating dynamic clustering of CFTR at cell membranes. Finally, we show in cells that overexpression of FLNa mutants defective in CFTR binding lead to decreased expression of CFTR band C, indicating incomplete processing of the CFTR protein.

955/B113
Treatment of Wounds with Insulin Followed by SDF1-A Accelerates Wound Healing.
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Insulin has become a molecule of interest for the treatment of wounds. In 1965-68, insulin was used to speed bone and cutaneous healing in rats and to treat wounds in diabetic mice. By 1998, insulin was used to treat burn wounds in rats and rabbits and in 1999, a clinical trial performed at UCLA found that insulin and zinc accelerate the wound healing process. More recently, we have shown that insulin activates Src, PI3K, Akt and SREBP through interaction with its receptor leading to improved re-epithelialization, angiogenesis and reduced scarring. Our findings point to insulin acting early during wound healing. It is also known that Stromal Derived Factor-1α (SDF1α), a chemokine that plays a key role in stimulating endothelial progenitor cell (EPC) migration from the bone marrow, potentially contributes to neovascularization, a critical step in wound healing. Therefore, we hypothesized that a combination of insulin in the first 4 days after wounding and SDF1α after that, would greatly improve the overall healing process. To test this possibility, we performed excision wounds in mice and applied insulin only, SDF1α only and insulin followed by SDF1α. We collected digital pictures of the wounds over the healing time and determined the wound area as percent of the original wound. In addition, the tissues were collected at 14 days, the time in which the wounds were closed, and prepared for histological sections. We show that in the early days of healing insulin alone accelerates wound closure, but insulin treatment followed by SDF1α accelerated the rate of re-epithelialization. Histological observations show that insulin increases the quality of wound healing, and SDF1α appears to provide more rapid epithelial wound coverage. In conclusion, our data suggest that using insulin followed by SDF1α is potentially a beneficial treatment combination for wound healing, including burn wounds.
Epithelial-to-Mesenchymal Transition: Role of Na,K-ATPase.


Epithelial-mesenchymal transition (EMT) is a process in which polarized epithelial cells undergo multiple biochemical changes and acquire a mesenchymal phenotype. EMT occurs during pathological processes such as cancer progression and fibrosis. Although, it is well recognized that EMT is an important event during cancer progression as well as in fibrotic disease, molecular mechanisms leading to EMT and target molecules affected during this process are poorly understood. A key molecule implicated in EMT is transforming growth factor-β (TGF-β1). This molecule induces EMT by Smad-dependent and independent pathways. Na,K-ATPase is a well-studied molecule for its role in ion homeostasis. We have shown previously that in addition to its epithelial ion transport function, Na,K-ATPase plays a fundamental role in the formation and maintenance of a well-differentiated, polarized epithelial phenotype in mammalian cells and that the Na,K-ATPase β1-subunit functions as a cell-cell adhesion molecule. In this study, using a cell culture model for cancer and fibrosis progression that responds to TGF-β1, we show that Na,K-ATPase is a target molecule of the TGF-β1 signaling pathways. We provide evidence that following treatment with TGF-β1 the surface expression of the β1-subunit of Na,K-ATPase is reduced, prior to well-characterized EMT markers and is associated with the acquisition of a mesenchymal phenotype. RNAi mediated knockdown confirmed the specific involvement of the Na,K-ATPase β1-subunit in the loss of the epithelial phenotype and exogenous over-expression of the Na,K-ATPase β1-subunit attenuates TGF-β1-mediated EMT. We further show that the levels of both the α1- and the β1-subunit of Na,K-ATPase are highly reduced in renal fibrotic tissues. These findings for the first time reveal that Na,K-ATPase is a target of TGF-β1-mediated EMT and is associated with the progression of EMT in both cancer and fibrosis.

Effects of Cellular Stretch on NFkB and STAT3 Activation in Human Intestinal Smooth Muscle Cells.

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Intestinal edema, which often occurs in trauma patients, causes slowed intestinal transit leading to delayed enteral feeding, prolonged hospital stays, and increased complications. Delayed intestinal transit due to intestinal edema has been attributed to decreased myosin light chain (MLC) phosphorylation leading to decreased intestinal contractile activity. Although the mechanism(s) of edema-induced decreases in MLC phosphorylation are unclear, NFkB and STAT3 are both activated and have been shown to play a role. We hypothesize that increased cellular stretch caused by edema development triggers NFkB and STAT3 activation that eventually leads to decreased MLC phosphorylation. Thus, the objective of this study was to determine the effects of cellular stretch on NFkB and STAT3 activation. We subjected primary human intestinal smooth muscle cells (HISMC) to either a control cyclical stretch program (CCS) as during basal intestinal contractile activity or an edema cyclical stretch program (ECS) consisting of an increasing cyclical stretch as observed in edematous intestine. Parameters for the cyclical stretch programs were based on In Vivo data obtained from our intestinal edema animal model. HISMC were also transfected with constitutively active STAT3 (caSTAT3) or IKK (calKK) (IkappaB Kinase). Activation of NFkB and STAT3 were significantly increased in cells subjected to ECS compared to CCS (CCS vs. ECS: P-stat3, 0.20 ± 0.09 vs. 0.34 ± 0.13 U/mg
protein; NFkB, 0.59 ± 0.22 vs. 0.96 ± 0.38). Transfection of unstretched HISMC with caSTAT3 or caIKK decreased carbachol stimulated MLC phosphorylation (MLC-P/MLC: Vector control, 1.76 ± 0.52; caSTAT3, 1.07 ± 0.31; caIKK, 1.05 ± 0.31). Furthermore, transfection with both caIKK and caSTAT3 significantly increased NFkB DNA binding activity. Inhibition of NFkB activation in stretched cells reduced STAT3 activation. These data indicate that increased cellular stretch can initiate the signaling cascades leading to decreased MLC phosphorylation and may be responsible for triggering intestinal edema-induced contractile dysfunction. In addition, the data suggests that NFkB and STAT3 interact to enhance both STAT3 and NFkB signaling.

958/B116
CD34 Enhances Myogenic Progenitor Cell Proliferation during Adult Skeletal Muscle Regeneration.
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Upon muscle damage, quiescent satellite cells activate, proliferate, and fuse into damaged myofibers to repair muscle. In chronic degenerative diseases, however, repair eventually fails. Cellular therapy has the potential of curing such diseases, but clinical trials have so far been disappointing. To improve current treatments, regulatory factors and mechanisms governing satellite cell function must be identified and better understood. Here, we focus on CD34, a commonly used satellite cell marker, in muscle regeneration. Although such a role has been proposed based on the tightly regulated expression of CD34 during satellite cell activation, no concrete evidence supporting this notion has yet been provided. Our experiments reveal a clear muscle regeneration defect in CD34KO mice following acute damage. Furthermore, cross-sectional area analysis shows a failure of CD34KO regenerating fibers to undergo hypertrophy. Because CD34 is expressed on a variety of cell types, we have optimized a technique to isolate a population of myogenic progenitor cells (MPCs) from skeletal muscle using flow cytometry. Our data suggests that CD34KO MPCs engraft with significantly less efficiency than WT controls, despite the fact that their differentiation remains normal. Moreover, Bromodeoxyuridine (BrdU) incorporation experiments show that CD34KO MPCs do not proliferate as efficiently as their WT counterparts. Altogether, these results support our hypothesis that CD34 has a role in the early stages of muscle regeneration. Specifically, we hypothesize that although CD34 is not completely necessary for MPC proliferation, CD34 significantly enhances this critical step. Currently, experiments are underway to depict the mechanism in which CD34 enhances proliferation.

959/B117
Anti-Inflammatory Role of Bone Marrow Stromal Cells Ameliorated the Irradiation-Induced Cell Death in the Cryptic Stem Cells of the Small Intestine.
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Radiation causes both sudden necrotic cell death and programmed apoptotic cell death. The immediate necrotic cell death may bring up strong inflammatory responses, which may then result in secondary cell death. In order to explore whether mesenchymal stem cells (MSCs) can ameliorate the radiation-induced inflammatory response and its deleterious effect on the tissue or not, mice were irradiated 8 Gy and MSCs were transfused just after the irradiation. Pro-inflammatory cytokines such as IL-1, IL6, and TNF-alpha were elevated by irradiation, which were nullified by MSCs transfusion. on the other hand, MSCs transfusion stimulated anti-inflammatory cytokine IL-10 in the blood. This anti-inflammatory effect was also confirmed by the suppression of neutrophils, eosinophils, and white blood cell counts. When TUNEL assay was performed in the frozen section of small intestine at 4.5 hours post-irradiation, apoptotic cell numbers were markedly decreased by MSCs transfusion, especially at the cryptic zone. Finally, we examined whether the MSCs transfusion in turn affect the regeneration from the radiation damage. The MSCs transfusion resulted in much faster regeneration after the irradiation. In conclusion, MSCs transfusion brings up anti-inflammatory response in the irradiated mice and thus reduce the radiation induced cell death.
**Host-Pathogen Interactions II (960 – 975)**

**960/B118**

**Echovirus 7 Uses a Dynamin- and Clathrin-Dependent Pathway to Infect Polarized Epithelial Cells.**

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Enteroviruses such as echoviruses and coxsackieviruses are human pathogens responsible for viral meningitis and myocarditis. They are transmitted via a fecal-oral route and are thought to cross the barrier presented by polarized epithelial cells in the gastrointestinal tract. Polarized cells have distinct apical and basolateral domains divided by tight junctions and these junctions also prevent the free flow of solutes and microbe across the epithelium. Previously, our group found that group B coxsackievirus 3 (CVB3) binds to decay-accelerating factor (DAF) on the apical cellular membrane of polarized epithelial cells (Caco-2), then moves to the tight junction from which it enters the cell by a mechanism that requires caveolin, but not dynamin. In this work, we studied the entry pathway of another DAF-binding enterovirus, echovirus 7 (EV7) by using dominant negative mutants, siRNAs, and drugs to block endocytic pathways. In contrast to what we observed for CVB3, infection by EV7 depends on dynamin and clathrin, but not caveolin. These results show that two related viruses, which bind to the same cell surface receptor, enter cells by different pathways.

**961/B119**

**A Role for Actin-Based Motility in Baculovirus Nuclear Translocation and Cell to Cell Spread.**

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Most viruses use the microtubule cytoskeleton for intracellular transport. Baculoviruses, including the type species *Autographa californica* M nucleopolyhedrovirus (AcMNPV), require intracellular transport to reach the nucleus for replication and the cell surface for spread, although previous work indicated that they do not require microtubules. Instead these viruses were shown to associate with actin, suggesting that they use an actin-based mechanism of transport. Using time-lapse microscopy, we observed that AcMNPV nucleocapsids rocketed through the cytoplasm by actin-based motility and were trailed by actin comet-tails. The compartmentalization of baculovirus actin-based motility differs from vaccinia virus, for which an extracellular form moves along the cell surface to promote cell-to-cell spread. AcMNPV actin-based motility depends on the nucleocapsid protein P78/83, an activator of the Arp2/3 complex, as a virus with a mutation in P78/83 showed impaired motility. One key function of actin-based motility is to promote nuclear entry. Moving viruses collided with and stuck to the nucleus, while their actin tails emanated from the point of impact in a corkscrew-like structure. Depolymerizing actin delayed and reduced nuclear entry, whereas depolymerizing microtubules had little effect. Following collisions with the nuclear periphery, nucleocapsids associated with nuclear pores, as revealed by immunofluorescence microscopy. Moreover, blocking nuclear pores by expressing a dominant negative derivative of importin β impeded viral nuclear entry, indicating that the major pathway of entry is through the pore. In addition to playing a role in nuclear translocation, actin-based motility served to translocate nucleocapsids into cell surface spikes, where they accumulated following the onset of viral gene expression. Movement was critical for transport to the surface because the P78/83 mutant shows a severe defect in its ability to localize to the cell periphery. Thus actin-based motility plays a key role in viral transit to the nucleus and the cell surface, and coordination between these transport processes is likely critical for promoting rapid cell-to-cell transmission during infection.
**962/B120**

**Respiratory Syncytial Virus Induces a Host Stress Response.**

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In this study we sought to determine the kinetics and the role of an integrated stress response in host cells during respiratory syncytial virus (RSV) infection. Background: RSV is a leading cause of serious viral lower respiratory tract illness in infants and the elderly worldwide. Unfortunately interactions between viral and host processes during replication have not been well characterized. Cytoplasmic RNA stress granules (SGs) are induced during specific stresses and contain mRNA, translation factors, and RNA binding proteins and have been proposed to selectively regulate host mRNA translation during the stress. Phosphorylation of the translation factor eIF2\(\alpha\) generally triggers SG formation. The host proteins G3BP and HuR are recruited to SGs and have been proposed to be important for SG formation and RNA stability respectively. Methods: Infections were performed using the RSV-A2 strain (MOI=1) for indicated times. SG formation and eIF2\(\alpha\) phosphorylation were determined by immunofluorescence. RNA silencing of G3BP and HuR was performed using shRNA lentiviral vectors. Viral titer and viral RNA levels were determined by plaque assay and RT-PCR respectively. All assays were performed using HEp-2 cells. Results: We observed increasing phosphorylation of eIF2\(\alpha\) and subsequent formation of SGs as RSV infection progressed. We also noted that while SGs were distinct from viral inclusion bodies the SG marker, HuR, was found in both structures. Cells with reduced G3BP expression were impaired for SG formation and regularly showed both a 10-fold reduction in RSV titers and viral RNA levels compared to wild type cells. Cells with reduced levels of HuR showed no difference in titer or viral RNA levels compared to wild type, SG formation was unaffected in these cells. Conclusions: Our findings demonstrate that RSV induces a robust stress response during infection. Inhibition of SG formation via G3BP shRNA results in a decrease of viral titers, indicating pro-viral role for the host response during infection. In addition, although the RNA binding protein HuR is found in RSV inclusion bodies, it does not appear to be essential for SG formation or viral replication.

**963/B121**

**Cellular Entry Mechanisms of Vaccinia Virus Extracellular Virions.**

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Vaccinia virus (VACV) is the prototypic poxvirus and a close relative of variola, the causative agent of smallpox. Considerable progress in understanding the entry route of its more abundant infectious form, mature virions (MVs), has been made in the last years. Despite their importance for virus spread within infected organisms, the entry mechanism of the less abundant extracellular virions (EVs) remains poorly characterized. Unlike most enveloped viruses, poxvirus EVs are surrounded by two lipid bilayers and therefore require a unique penetration process. EV infection presumably involves the non-fusogenic disruption of the outermost membrane followed by fusion of the inner membrane with cellular lipid bilayers. We used recombinant VACV (strain IHD-J) encoding EGFP or EGFP fusion proteins to characterize the entry process of EV particles into human epithelial cells by a combination of fluorescence microscopy and flow cytometry. Our results indicate that EV particles are endocytosed in the course of the entry process. Infection requires actin dynamics as well as activity of P21/Cdc42/Rac1-activated kinase 1 (PAK1) and Na\(^+\)/H\(^+\) exchangers, suggesting a macropinocytotic mechanism. Furthermore, EV infection is dependent on the acidification of endosomal vesicles. We find that low pH-treatment disrupts the outer EV membrane In Vitro and partly rescues infection in the presence of the vacuolar H\(^+\)-ATPase inhibitor Bafilomycin A1, a potent inhibitor of endosomal acidification. EV particles of the VACV strain Western Reserve are less sensitive to inhibitors of endosomal acidification; a fraction of the EV particles might therefore enter cells by an alternative entry pathway, possibly involving glycosaminoglycan-dependent disruption of the EV wrapper at the plasma membrane.
In conclusion, our data suggest VACV EV particles are internalized by a process that resembles macropinocytosis. Subsequent acidification of endosomal vesicles triggers disruption of the outermost membrane and thus exposes the viral fusion machinery on the inner membrane. Fusion of the inner lipid bilayer with limiting cellular membranes releases the viral core with the genome into the host cell cytoplasm.

964/B122
From Target Identification to Drug Discovery: The HIV-1 Integrase/Human LEDGF-Case Study.

More than 25 years after its discovery, Human Immunodeficiency Virus 1 (HIV-1) is still one of the major threads of human life. Most anti-HIV drugs currently in use inhibit the catalytic activities of viral enzymes. However, novel mechanisms of action are needed to cope with the increasing resistance against these drugs. A promising alternative is to target the complex interplay between viral proteins and host cells. We used Hybrigenics' ULTImate Y2H™ technology to perform a large-scale yeast two-hybrid study with all HIV-1 proteins as baits, using highly complex cDNA libraries from the CEMC7 T cell line. Analysis of the interaction network led to the selection and functional validation of several protein interactions. We will detail the in-depth characterization and chemical targeting of the interaction between HIV-1 integrase and the human transcriptional co-activator LEGDF (1). siRNA knockdown of LEDGF demonstrated the functional importance of this interaction for targeting integrase to the chromosome. Using a random-primed HIV-1 library, the integrase catalytic core was mapped as the smallest interacting domain in a rebound yeast two-hybrid screen with LEDGF as bait. Moreover, by screening loss-of-affinity mutants in yeast, Q168 of integrase was identified as one of the hot-spots of the interaction. Based on these functional and detailed molecular evidences, we decided to target the HIV-1 integrase / human LEDGF interaction to identify new molecules with therapeutic use in HIV treatment. We set up an assay for High Throughput Screening based on the HTRF® technology by Cisbio International. 77,000 small-molecule compounds were screened for their ability to inhibit the interaction and several profiles of compounds were identified, pointing to different options for hit to lead optimization. This approach highlights the straightforward way from a complex protein interaction network to the functional validation of a selected interaction as therapeutic target, eventually leading to the identification of small molecules as promising new antiviral drug candidates. (1) S. Emiliani et al. (2005) J. Biol. Chem. 280 : 25517-23

965/B123
The Role of Autophagy during Coxsackievirus Infection in Neural Stem Cells.
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Recently the process of autophagy has been identified as a crucial step for the replication and survival of viral pathogens following infection of the cell. Some viruses have been shown to manipulate the autophagic process in order to efficiently replicate within the cell, rather than fall prey to this catabolic process and be destroyed in the lysosome. As with other picornaviruses, coxsackievirus B (CVB) has been identified as a virus that utilizes autophagy to its advantage following infection. However, few studies have determined if virus-induced autophagy occurs following infection of stem cells. Therefore, we studied the role of autophagy following CVB infection in neural stem cells, which we previously identified to be highly susceptible to CVB infection, both in culture and in vivo. Neurospheres, or free-floating structures generated by neural stem cells in culture, can remain undifferentiated, but also have the ability to differentiate into all three cell lineages of the central nervous system, including neurons, astrocytes, and
oligodendrocytes. We measured autophagic induction by utilizing LC3-GFP to label autophagosomes following infection with a recombinant dsRED coxsackievirus B3 (dsRED-CVB3). No change in the level of autophagy was seen in undifferentiated neurospheres following infection with dsRED-CVB3. However, a significant decrease in the level of autophagy was observed in differentiated neural stem cells following dsRED-CVB3 infection. In particular, β-tubulin positive differentiated neurospheres underwent the greatest decrease in autophagy of the three cell lineages. In contrast to the results seen with neurospheres, HL-1 cells, a transformed cardiomyocyte cell line, showed an increase in the level of autophagy following dsRED-CVB3 infection. Furthermore, viral titers in HL-1 cells decreased in the presence of an inhibitor of autophagy (3-MA), while viral titers increased in the presence of an inducer of autophagy (CCPA). Hence, we conclude that the role of autophagy in modulating CVB replication appears cell type-specific.

966/B124  
**microRNA Fingerprints in Venezuelan Equine Encephalitis Virus Infection.**  
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Background: MicroRNAs (miRNA) are small endogenous RNA (~22nts) molecules and play an important role in regulating gene expression, mainly by the mechanism of mRNA degradation or translational repression. MiRNAs are important in normal as well as abnormal cellular processes like development, differentiation, cell proliferation, tumorigenesis, neuronal development and hematopoiesis. Cellular miRNA plays an important role in the pathogenesis of viral infections including hepatitis c virus, human cytomegalovirus and human immunodeficiency virus. Venezuelan equine encephalitis virus (VEEV) is a member of the alphavirus genus in the family Togaviridae. Alphaviruses are mosquito-borne and may cause fatal human diseases. The genome consists of an 11.4kb, positive sense, single stranded RNA. VEEV can be spread by aerosol and has been weaponized making it a potential biothreat agent. Methods: 5-6 week old CD-1 mice were infected with 1000 pfu of V3000 in right footpad. Total RNA was isolated from brains of the infected and uninfected mice using TriZol reagent at different time points post infection (p.i). Brain samples from uninfected mouse were used as an experimental control for basal level expression of miRNA. The miRNA expression profile was generated by quantitative real-time PCR (qRT-PCR) with ABI 7900HT Fast Real time PCR system using a multiplex pool of miRNA stem-loop primers. Statminer software (Integromics Inc) was used for determining the statistically significant differentially miRNAs. Results: Data indicated significant modulation of more than 10 miRNAs. Studies are in progress to analyze miRNA expression profile at different p.i time points. These microRNA are being analyzed for their targets to delineate their role in the regulation of downstream cellular pathways. Conclusions: These results suggest that miRNA may play an important role in the pathogenesis of VEEV infection by affecting downstream gene signaling pathways. This study will help in finding novel biomarkers for VEEV infection and will also allow us to design novel antivirals and miRNA based vaccines for VEEV. These studies were supported by JSTO-CBD/DTRA Contract/ Grant/ Intergovernmental Project Order/ Project # 4.10019_07_US_B.

967/B125  
**Disruption of the Toll-Like Receptor 4 Signaling Pathway by Salmonella Effector SigD.**  
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The enteropathogenic bacteria *Salmonella* are the main cause of food-borne gastroenteritis and typhoid fever worldwide. To evade the host immune response, *Salmonella* invades mammalian cells and survives within an intracellular niche, the *Salmonella* containing vacuole (SCV). The activation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) triggers an immune response
to counter infection. The stimulation of this pathway requires the recruitment of adaptor proteins, TIR-associated protein (TIRAP) and TRIF-related adaptor molecule (TRAM). Recruitment and activation of these molecules is dependent on the membrane lipid, phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2). The Salmonella effector Salmonella invasion gene D, SigD, is a 4-phosphatase that depletes PI(4,5)P2 from the host cell and plasma membrane during invasion. Thus, in this study, we investigated if SigD could lead to the disruption of the TLR4 pathway. We observed that SigD expression caused the disappearance of TIRAP from the SCVs in HeLa cells. Assessment of the myeloid differentiation primary response gene 88 (MyD88) dependent pathway showed that the expression of SigD attenuates the degradation of IκBα in wild type (wt) Salmonella-infected cells. The TLR4 MyD88 independent pathway requires the adaptor protein TRAM. SigD did not affect the localization of TRAM in the SCV. However, the activation of protein kinase C (PKC) ε, which is required for TRAM activation, is reduced in HeLa cells invaded with wt bacteria in comparison to the SigD deficient Salmonella strain. Taken together, these results suggest that SigD can disrupt both branches of the TLR4 signaling pathway.

968/B126
Legionella pneumophila Uses β1 Integrins to Adhere to Lung Epithelial Cells.
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Legionella pneumophila, the etiological agent of Legionnaires disease is an intracellular pathogen. The interactions between this respiratory pathogen and host macrophages have been extensively studied but more than 95% of the human lung surface is covered by epithelial cells. Therefore, these cells present an important niche for the intracellular survival of the bacteria. It has been shown that L. pneumophila serogroup 1 (Lp1) invades lung epithelial cells but the mechanisms of adhesion and invasion of these cells are poorly described. Our objective is to investigate the mechanisms used by Lp1 to adhere to lung epithelial cells and the downstream signalling pathways that are activated to initiate bacterial internalization. Using scanning electron microscopy we have characterized that the adhesion of L. pneumophila to lung epithelial cells occurs by the ‘zipper’ mechanism. The host cell membrane wraps around the adhering bacteria, forming a membrane rich structure we are referring to as a ‘pocket’. We have utilized fluorescent chimeras and immunostaining techniques for confocal microscopy to demonstrate that Lp1 adheres to lung epithelial cells (NCI-H292) using the host cell β1 integrin receptors. Engagement of these receptors initiates an adhesion process that is dependent on actin polymerization and the recruitment of actin motor Myosin II. These results have been further confirmed through biochemical assays with pharmacological inhibitors. We have also shown that the adhesion of Lp1 to NCI-H292 cells causes the activation of the focal adhesion signalling pathway and the recruitment of focal adhesion proteins talin and focal adhesion kinase. Based on our results, we conclude that β1 integrin receptors mediate the adhesion of L.pneumophila to lung epithelial cells. Adhesion to these receptors causes bacterial internalization by the ‘zipper’ mechanism through the focal adhesion signalling pathway.

969/B127
Characterization of the Roles of the LRR Proteins InlJ and InlH in the Virulence of L. monocytogenes.
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Using comparative genomics, our group identified several potential new virulence factors of Listeria monocytogenes. Subsequent In Vivo assays characterized InJ as a virulent factor. Like the major listerial invasion proteins InlA and InlB, both InlJ and previously identified virulence factor, InlH, are members of the internalin family of proteins characterized by the presence of
leucine-rich repeat domain at the N-terminal. Unlike inlA and inlB, inlJ is only expressed by L. monocytogenes upon infection in vivo. InlH expression is induced by stress stimuli and in the host. To date little is understood about their function in virulence. In order to better understand the listeriosis pathophysiology, we are investigating both InlH and InlJ mechanisms of action. Interestingly, in vivo, InlH down-regulates the IL6 level without affecting the level of neither Tnf nor IL1. Currently, we are working on understanding the role of InlH in the specific regulation of IL6 and the modulation of innate immunity. When expressed on the surface of the avirulent L. innocua InlJ has a high bacterial adherence property. However, the recombinant InlJ protein does not bind cells in vitro. To understand InlJ function, we used comparative DNA Microarray to identify the signaling cascade that InlJ may modulate during infection. Our preliminary data suggest that InlJ alters the phosphoinositide 3-kinase (PI3K) pathway in the host, by down-regulating the PTEN gene. We are also investigating the expression pattern and localization of InlJ in infected organs to decipher its local function.

970/B128
Tissue-Specific Activities of SARM-ASK1-MKK3 Signaling Coordinate Immunity and Behavior to Pathogenic and Nutritional Bacteria in C. elegans.
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Microbes represent both an essential source of nutrition and a potential source of lethal infection to the nematode Caenorhabditis elegans. Immunity in C. elegans requires a signaling module comprised of orthologs of the mammalian Toll-Interleukin-1 Receptor (TIR) domain protein SARM, the mitogen-activated protein kinase kinase kinase (MAPKKK) ASK1, and MAPKK MKK3, which activates p38 MAPK. We determined that the SARM-ASK1-MKK3 module has dual tissue-specific roles in the C. elegans response to pathogens—in the cell autonomous regulation of innate immunity, and the neuroendocrine regulation of serotonin-dependent aversive behavior. SARM-ASK1-MKK3 signaling in the sensory nervous system also regulates egg-laying behavior that is dependent on bacteria provided as a nutrient source. Our data demonstrate that these physiological responses to bacteria share a common mechanism of signaling through the SARM-ASK1-MKK3 module and suggest the co-option of ancient immune signaling pathways in the evolution of physiological responses to microbial pathogens and nutrients.

971/B129
VIP Attenuates Loss of Epithelial Barrier Function Induced by EPEC via VIP/Calmodulin Complex Formation.
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The gastrointestinal tract is lined by a single layer of epithelial cells connected by intercellular junctions located between individual cells, the most apical of which is the tight junction. The tight junction consists of transmembrane proteins (e.g. claudins, occludin, tricellulin and JAM) and cytoplasmic plaque proteins (e.g. ZO 1-3). A healthy epithelium provides a physical barrier between luminal contents and the underlying milieu. Enteric pathogens such as Enteropathogenic E. coli (EPEC) disrupt the colonic epithelial barrier causing diarrhea and intestinal inflammatory disease. The epithelial barrier responds to stimuli from the enteric nervous system; studies have shown that vasoactive intestinal peptide (VIP) ameliorates EPEC-induced barrier disruption. In this study, colonic monolayers were treated with VIP and the mechanisms of action assessed. Transepithelial Resistance: Confluent Caco-2 monolayers were seeded onto Transwell™ filters and basally treated with VIP (10-6M), Forskolin (10-7M), SQ22536 (200μM) and TFP (10-5M) prior to infection. Resistances were monitored for 4 hours post-infection. Immunocytochemistry: Caco-2 monolayers were fixed after 4 hours and labelled with ZO-1, Claudin-3 and tricellulin. Western Blotting/Co-immunoprecipitation: Caco-2 lysates were analysed via SDS-PAGE and probed for MLCK, Calmodulin/MLCK complexes, MLC, MLC-p. EPEC infection disrupts epithelial barrier function by decreasing resistance and disrupting ZO-1, claudin-3 and tricellulin distribution. VIP treatment protects the epithelial barrier, preventing tight junction disruption and
maintaining normal barrier function. Disruption of the epithelial barrier is concomitant with increased MLCK expression (1.5-fold, n=3, p<0.01) and MLC-p phosphorylation (1.4-fold, n=3, p<0.01). Stimulation or inhibition of cAMP had no effect on barrier resistance during infection. Inhibition of calmodulin prevented MLCK-induced barrier disruption (p < 0.01, n=4). VIP/calmodulin complex formation prevents EPEC-induced increase in MLCK activity VIP protects barrier integrity during EPEC infection via a cAMP-independent pathway and prevents MLCK-induced disruption of barrier function via sequestration of calmodulin.

972/B130
Membrane Protein Dynamics in Mature Phagosomes.
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Nascent phagosomes undergo successive fusion and fission events to acquire microbicidal components from endocytic compartments, a process known as maturation. Fully mature phagosomes, or phagolysosomes, have constant levels of lysosomal membrane proteins, and their size appears constant. These observations suggest that phagolysosomes are static compartments. On the other hand, fluid phase markers added to cells after maturation is complete gain access to phagolysosomes, indicating continuous influx of endocytic material. Steady-state maintenance of phagolysosomal size and composition must therefore involve continuous removal of membranous and soluble material. We used fluorescence recovery after photobleaching (FRAP) and photoactivatable GFP (PA-GFP) to study the kinetics of lysosome-associated membrane protein 1 (LAMP-1), a protein characteristic to late endosomes/lysosomes that is acquired by phagosomes following fusion with lysosomes. We have found that LAMP-1 continually enters and exits the mature phagolysosome, suggesting that this compartment is not static but is instead in a dynamic steady state. We are currently investigating the mechanistic details of this process.

973/B131
Infection and Translocation of Bacillus anthracis across Lung Epithelium.
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Inhalational anthrax is initiated by the entry of Bacillus anthracis spores into the lung. A critical early event to establish the infection is the dissemination of bacteria from the lung. Our In Vitro and In Vivo data suggested that B. anthracis could directly cross the lung epithelium. We found that B. anthracis could adhere to and be internalized by cultured lung epithelial cells. Using a mouse model, we presented evidence that B. anthracis spores were taken up by lung epithelial cells In Vivo soon after spores were delivered into the lung. In transwell assays, B. anthracis were able to translocate across an A549 cell barrier without disruption of the barrier integrity, suggesting a transcellular migration route. To further understand how B. anthracis passages through lung epithelial cells, we further analyzed the intracellular trafficking and fate of internalized spores in lung epithelial cells. Internalized spores were able to survive and persist within lung epithelial cells. In contrast, germinated spores and vegetative bacilli preferentially exited the cells in a process that did not involve cell lysis, suggesting a bifurcation of fate for dormant spores and vegetative bacilli in lung epithelial cells. Investigations to understand the mechanisms underlying the trafficking pathway of spores and vegetative bacilli are currently underway.

974/B132
Microbicidal and Immunomodulatory Properties of SALF23 Peptide Designed from LPS Binding Domain of Scylla Serrata Anti-LPS Protein.
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Introduction and hypothesis The bacterial lipopolysaccharide (LPS) is known to be a major initiator of systemic inflammatory cascade and is regulated by cytokines particularly TNF-α. We have recently an anti-LPS (ALPS) protein from the hemocytes of the mud crab, Scylla serrata (Fish Shellfish Immunol. 2009 27(2):275-84). In this study we have evaluated a synthetic peptide, SALF23, designed from LPS binding domain of S. serrata ALPS protein, for its immunomodulatory properties. Methodology and results SALF23, corresponding to residues 28-51 of S. serrata ALPS was synthesized and was shown by a MIC assay to demonstrate a significant antibacterial activity against E. coli, P. aeruginosa, S. aureus and S. pyogenes at concentrations 25 - 100 μg ml-1. A microtiter plate based LPS binding assay of peptide demonstrated a significant binding at 100 μg ml-1 and above. Upto 2 folds decrease in TLR-4 transcripts was observed in U937 cells incubated with LPS along with SALF23 peptide (200 μg ml-1) as observed by real time PCR. Similar pattern was observed for TNF-α as measured by ELISA. SALF23 (100-200 μg ml-1) also caused a 45-60% decrease in TNF-α regulated HIV-LTR promoter activity in a TZM-bl cell line assay. Conclusion and significance Sexual transmitted pathogens and their associated syndromes have been shown to cause severe inflammation in the female reproductive tract (J Immunol, 2002, 168: 2424-32). Cytokines secreted during this inflammatory cascade, particularly TNF-α, is known to increase HIV infection in such conditions (J Immunol, 2001, 166: 2342-47). from the above studies it is clear that SALF23 peptide can bind to LPS and inhibit its further interaction with TLR-4, thereby down regulating TNF-α production and subsequent decrease in TNF-α regulated HIV infection. In conclusion, SALF23 peptide can be considered to be a molecule of high therapeutic value.

975/B133
Cytokinesis Failure Causes Multinucleation in Chlamydia trachomatis Infected Cells.
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Chlamydiae are obligate intracellular bacteria, which live in a membrane-bound vacuole called an inclusion. Chlamydia trachomatis is the cause of the most prevalent STD in the United States. There are often no initial symptoms, leading to chronic infections causing pelvic inflammatory disease and infertility. on a cellular level, infection with C. trachomatis has been shown to cause changes in host cells such as multinucleation. The two main mechanisms that can initiate multinucleation are cell fusion and failure in cytokinesis. Our goal was to determine which mechanism was the cause of multi-nucleation of host cells during infection by chlamydia. Using confocal microscopy, we were able to confirm infected cells fail in cytokinesis while eliminating the possibility of cell fusion. Since cytokinesis failure is the main cause of multinucleation, we choose to look at the effects of chlamydia on the stages leading up to cytokinesis during mitosis. Our findings show that 12 hours after infection, we begin to see a higher ratio of cells in prometaphase than metaphase compared to uninfected cells suggesting chlamydia is interfering with the cells ability to properly align the chromosome on the metaphase plate. for these cells to progress through mitosis, the infected cells would need to delay exit from metaphase until proper alignment is achieved. However, we found that the mitotic index (a measure of the relative time cells take to complete mitosis) of infected cells instead decreased, suggesting that infected cells spent less time in mitosis. We therefore hypothesize infected cells fail to align their chromosomes properly before proceeding to anaphase resulting in a higher proportion of cells failing in cytokinesis leading to multinucleated cells.

Actin Associated Proteins II (976 – 989)

976/B134
Regulation of the Actin Cytoskeleton Downstream of Guidance Receptors: The Multi-Domain Redox Enzyme Mical Is a Direct Regulator of Actin Dynamics.
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Semaphorins are one of the largest families of extracellular guidance cues and are widely studied for their effects on cell movement, morphology, angiogenesis, immunology, and cancer. Interestingly, semaphorins and their cell surface plexin receptors dramatically alter actin cytoskeletal dynamics but the molecules connecting semaphorins and plexins to these cytoskeletal elements are unknown. Recently, we and others have identified that members of the MICAL family of multidomain Redox enzymes are important for Semaphorin/Plexin signaling but the biochemical role of MICAL proteins in mediating these events has remained elusive. We now find using well-established In Vivo and In Vitro assays that MICAL is both necessary for proper actin cytoskeletal organization In Vivo and sufficient to reorganize the actin cytoskeleton In Vivo and in vitro. In particular, we find that MICAL proteins are necessary for normal F-actin organization and bundling during development and require their Redox domain for these functions. Elevating MICAL levels In Vivo in a Redox dependent manner is sufficient to disassemble bundles of actin filaments and reorganize parallel F-actin bundles into a meshwork of short, branched actin filaments. MICAL physically associates with filamentous actin and thereby provides the conduit between the semaphorin cell surface receptor, Plexin, and the actin cytoskeleton. Utilizing In Vitro actin biochemical assays with purified MICAL protein we also find that MICAL directly alters actin dynamics by limiting actin polymerization and inducing the disassembly of actin filaments. These observations reveal a novel role for MICAL in directly regulating actin dynamics and also identify the MICAL Redox enzymatic domain as a novel F-actin disassembly module. Together, these results also indicate that specific Semaphorin/Plexin guidance receptor-mediated reorganizations of the F-actin cytoskeleton can be precisely achieved in space and time through MICAL.

977/B135
Use of Camelid Single Domain Antibodies (Nanobodies®) as Inhibitors of Structural Cytoskeletal Proteins (Gelsolin, L-Plastin).
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Invasion and metastasis of cancer cells relies in part on dynamic reorganization of the actin cytoskeleton which is the driving force for cellular motility. Actin binding proteins aid in this process by virtue of their ability to interact reversibly with actin and actin filaments. The expression level of quite a few actin associated proteins is upregulated in cancer cells, and many studies have demonstrated a correlation between their expression level and cancer cell motility and/or invasion In vitro and in vivo. We developed a new instrument to investigate the contribution of actin binding proteins in cancer cell invasion and metastasis: Camelid single domain antibodies (VHHs, nanobodies®) are small, monovalent, monoclonal antibodies that can be easily cloned. Nanobodies® against gelsolin and L-plastin were used as intrabodies with the aim of inhibiting distinct biological activities of these structural proteins without affecting their expression level in cells. We show that selected nanobodies® bind their target with nanomolar affinity. Intracellular expression of L-plastin nanobodies® leads to inhibition of filopodia formation in PC-3 cells. Interestingly, gelsolin nanobodies® are able to discriminate between different populations of gelsolin in cells (i.e. actin bound or not). Importantly, expression of nanobodies® in cancer cells reduces motility and In vitro invasion of these cells to the same extent as siRNA. This approach allows analysis of the role of (actin associated) proteins in tumorigenesis at the level of the protein. Conclusion: Nanobodies® are stable in the cytoplasmic environment of eukaryotic cells and represent a potent tool to curb protein function without manipulating gene expression. This methodology can be extended in principle to any protein.

978/B136
Identification of Conserved Actin-Binding Surfaces on Coronin: Role of Actin Binding in Regulating Actin Dynamics and In Vivo Localization.
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Coronin is a highly conserved actin-binding protein that functions closely with both Arp2/3 complex and ADF/cofilin to govern cellular actin dynamics. Despite the importance of coronin in regulating a wide range of actin-based processes, a detailed understanding of its molecular interactions with actin has been lacking. Here, we performed a structure and function analysis of the yeast coronin (Crn1) β-propeller domain, which binds to F-actin and is conserved in all coronin family members. Using site-directed mutagenesis, we generated 22 new alleles, targeting coronin’s functional residues. Three alleles severely impaired F-actin binding, crn1-2, crn1-6, and crn1-20; six other alleles showed mild-to-moderate defects. The key actin-binding surfaces form a belt on the β-propeller structure. Crn1 polypeptides carrying these mutations failed to synergize with ADF/cofilin in vitro, and diminished Crn1 localization to cortical actin patches in vivo. Thus, coronin localization and function depend on direct interactions with F-actin. The conservation of the actin-binding residues across distant species and in all three major classes of mammalian coronin suggests that the nature of the interaction with actin, and possibly the effects on F-actin dynamics, may be conserved in distant coronin family members.

979/B137
CLIC5a Maintains the Ezrin-Dependent Podocyte Architecture.
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We found that CLIC5A mRNA and protein are highly enriched in kidney glomeruli. CLIC5A is a component of ERM (ezrin-radixin-moesin)/actin complexes. In renal glomerular podocytes, phospho-ezrin mediates the interaction of podocalyxin with the actin cytoskeleton and its disruption produces foot process effacement and proteinuria. By confocal immunofluorescence (cIF) and immunogold transmission electron microscopy CLIC5A localizes to the apical domain of glomerular podocytes with the same distribution as ezrin and podocalyxin. By cIF, we find exquisite colocalization of CLIC5A with ezrin and podocalyxin in glomeruli. CLIC5A immunoprecipitates prepared from lysates of purified mouse glomeruli contained podocalyxin. In jitterbug (jbg/jbg) mice, which are deficient in CLIC5A due to a partial gene deletion, the podocyte foot processes are much broader than in wild-type (+/+) mice and this is associated with microalbuminuria. Western blots were performed on lysates of 20 microdissected glomeruli from 3 each, wild-type (+/+), heterozygous (+/jbg) and CLIC5A deficient (jbg/jbg) mice. CLIC5A was reduced in +/jbg and absent from jbg/jbg glomeruli. Ezrin abundance was dramatically reduced in jbg/jbg, and also in +/jbg mice, compared to +/+ mice, while podocalyxin abundance did not differ between the groups. Similarly, by cIF, ezrin and phospho-ezrin abundance in podocytes was markedly diminished in jbg/jbg compared to +/+ mice. To determine whether CLIC5A deficient mice are more vulnerable to glomerular podocyte injury than +/+ mice, they were injected IV with adriamycin, and albuminuria was quantified 3 weeks later. In 2 pairs of age-matched male mice, 10 mg/kg adriamycin induced 11.7 and 14.1 fold greater albuminuria in jbg/jbg than in +/+ mice. In 2 pairs of age-matched female mice injected with 15 mg/kg adriamycin, there was 3.6 and 1.8 fold greater albuminuria in jbg/jbg than in +/+ mice. Hence, CLIC5A is a component of the podocalyxin/ezrin complex in renal glomerular podocytes, and its absence leads to decreased ezrin, and phospho-ezrin abundance in these cells. Consequently, podocyte architecture is highly abnormal and susceptibility of podocytes to adriamycin-induced injury is increased.

980/B138
Expression of Formins during In Vivo and In Vitro Cardiomyocyte Differentiation.
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Cardiac repair by cell replacement therapy is a promising approach for the treatment of end stage heart failure. Pluripotent stem cells can be differentiated into cells that are excitable and capable of generating mechanical force. In order to successfully engraft in vivo, individual cells must integrate into the mechanical and electrical network of the host myocardium. Expression of formin
proteins has been shown to isoform-specifically influence adherens junction formation. Objective: to identify formin isoforms expressed during In Vivo and In Vitro differentiation of mouse cardiomyocytes. Methods: In Vivo cardiogenesis was analyzed in samples of neonatal cardiomyocytes and ventricular tissue isolated from neonatal or adult mice. In Vitro differentiation was studied by differentiating W4 mouse embryonic stem (ES) cells (129S6 genetic background) into contracting cardiomyocytes. This was accomplished by 3 day aggregation of undifferentiated ES cells into embryoid bodies by the hanging drop method, 5 days of suspension and 3 weeks of adhesion culture. Relative transcript amount analysis was carried out by RT-PCR; protein expression was analyzed by immunoblot and immunofluorescence analysis. Results obtained: mDia was expressed at high transcript and protein levels in undifferentiated ES cells. Transcript expression decreased during In Vivo and In Vitro differentiation; this was verified by reduction of mDia protein in cardiogenic embryoid body derived outgrowths (29%), neonatal (37%) and adult ventricle (5%). Low amounts of mFhod1, mFhod3 and mDia3 transcript were detected in ES cells. Transcript and immunofluorescence data for mFHOD1 revealed increased expression in neonatal cardiomyocytes and In Vitro differentiated cardiomyocytes, whereas mFHOD3 and mDia3 displayed high expression in neonatal and adult ventricular tissue. Summary: 1. We detected an isoform specific expression profile of formins during cardiogenesis. 2. The formin profile of In Vitro differentiated cardiomyocytes resembled neonatal and not adult cardiomyocytes. Conclusions: Modifying the expression of formins towards the profile in the adult ventricular cardiomyocytes could improve cell engraftment in stem cell based cardiac repair strategies.

981/B139
Regulating Actin Filament Bundling by Lim Proteins.
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From the various LIM-domain containing proteins present in animals, only CRPs have counterparts in plants, suggesting that this subset is involved in basic and conserved eukaryotic cellular processes. Noticeably, defects in CRP activities are associated with severe illness, e.g. chronic heart failure, which may be attributed to cytoskeletal disorganization/dysfunction. These last years we provided evidence that plant LIM proteins regulate the actin cytoskeleton organization and dynamics by inducing the formation of actin bundles/cables. Here we present key data regarding the expression, activity and regulation of an entire plant LIM protein family. According to their expression pattern, the six Arabidopsis LIM proteins divide into two subfamilies: the pollen and non-pollen LIM subfamilies. In Vitro assays indicate that the corresponding proteins all retain actin-bundling activity. However, they are differentially regulated by pH and calcium. Indeed, only the pollen subfamily members are inhibited by an increase of pH and/or calcium whereas the non-pollen subfamily members are not responsive to these factors. Importantly, In Vitro data are confirmed by In Vivo experiments conducted in Arabidopsis cells whose cytoplasmic pH and calcium concentration were artificially modified. Noticeably, results are consistent with a role of plant LIM proteins in the positioning/maintaining of actin bundles in developing pollen tubes. Indeed, the high pH and calcium concentration reached in the pollen tube apex would inhibit LIM protein bundling activity and prevent the formation of actin bundles in this region. In contrast, the lower pH and calcium concentration found in the pollen tube shank would “activate” LIM proteins and therefore trigger the formation of stable actin bundles.

982/B140
Actin Binding Domain of Drebrin Affects Lateral and Longitudinal Contacts in F-Actin.
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Drebrin a is a brain specific F-actin binding protein involved in organizing the dendritic pool of actin. Drebrin shares homology with the cofillin family proteins through an N-terminal ADF-homology domain (dADFhhd). It also contains the actin-binding domain (DrABD, residues 233-300/317), which was shown to cause similar In Vivo rearrangements in actin cytoskeleton as the full-length protein. In this study, we examined the structural effects of different drebrin constructs
on actin filaments. As estimated by pelleting experiments, isolated AFD homology domain of drebrin has very low, if any, affinity to F-actin. Mutations mimicking N-acetylation and phosphorylation in the C-terminal part of the drADFhed did not improve its binding to F-actin. Site directed mutagenesis combined with chemical cross-linking was employed to probe the effects of isolated DrABD on lateral and longitudinal contacts in F-actin. Yeast actin mutant S265C was chosen to assess the effect of DrABD on the lateral contacts in actin while mutant Q41C was employed to probe the longitudinal contacts. DrABD enhanced the rate of interstrand disulfide cross-linking between Cys265 and Cys374 in S265C F-actin. In contrast, the inhibition of disulfide cross-linking between residues 41 and 374 on actin was documented for the mutant Q41C. Our results suggest that drebrin induces conformational changes in F-actin's structure.

983/B141
Characterization of a Novel Adaptor Protein: AFAP1-L1.
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The actin-filament associated protein (AFAP) family of adaptor proteins consists of three members: AFAP1 (AFAP-110), AFAP1-L1, and AFAP1-L2 (XB-130) with AFAP1 being the best described as a Src binding partner and actin cross-linking protein. A homology search of AFAP1 recently identified AFAP1-L1, which has similar domain structure and cellular location. As an adaptor protein, AFAP1-L1 consists of one SH3 binding motif, two SH2 binding motifs, two pleckstrin homology domains, a substrate domain for serine/threonine kinases, a putative leucine zipper and a putative actin binding domain. AFAP1 contains two juxtaposed SH3 binding motifs and binds Src using the N-terminal motif. AFAP1-L1 has only one SH3 binding motif and lacks the sequence known in AFAP1 to bind Src. AFAP1-L1 is not thought to be a Src binding partner but may form interactions with proteins through its SH3 binding motif that differ from AFAP1 as witnessed through a panomics array. In addition to activating Src, AFAP1 can cross-link actin by binding through its actin binding domain and contacting other AFAP1 molecules through a leucine zipper. AFAP1-L1 also contains a putative actin binding domain and a less-defined leucine zipper. As AFAP1-L1 has been shown to localize to actin filaments, this putative leucine zipper may allow AFAP1-L1 to multimerize with other AFAP1-L1 or AFAP1 molecules and also cross-link actin. AFAP1-L1 has been shown by fluorescent microscopy to decorate actin filaments and move to punctate actin structures and colocalize with cortactin upon stimulation. Immunohistochemical analysis of AFAP1-L1 shows it to be expressed at higher levels in tissue such as muscle when compared with AFAP1. AFAP1-L1 also appears to have differential expression from AFAP1 in the brain. AFAP1-L1 is highly upregulated in multiple cancers while AFAP1 is weakly upregulated. Due to structural similarity with AFAP1, we hypothesize that AFAP1-L1 has a role in actin filament rearrangement. Because it lacks the SH3 binding motif responsible for binding Src and has differential expression, AFAP1-L1 is thought to relay these signals for actin rearrangement through domain or binding partners that differ from AFAP1.

984/B142
A Novel Role for Coronin 2A in the Formation of Cofilin-Actin Rods.
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In response to environmental stress, such as ischemic injury or ATP-depletion, cells have developed protective mechanisms to promote cell survival. One such mechanism, the formation of Cofilin-Actin rods, may reduce ATP consumption by approximately fifty percent by reducing actin turnover. Previously, Cofilin and Actin were the only known proteins found in stable Cofilin-Actin rods. Here, we show that ectopic expression of Coronin 2A induces the formation of aggregates similar to Cofilin-Actin rods. Ectopic Coronin 2A induced rods contain Cofilin and Actin, but unlike ATP-depletion induced Cofilin-Actin rods, they stain positively with phalloidin and can incorporate fluorescent-LifeAct peptide. Previous fluorescence recovery after photobleaching (FRAP) experiments demonstrated that ATP-depletion induced Cofilin-Actin rods contain very
stable, immobile Cofilin. Our FRAP analysis however, indicates that Coronin 2A induced rods contain mobile Cofilin and immobile Coronin 2A. Ectopic expression of Coronin 2A also potentiates Cofilin-GFP rod formation upon ATP-depletion without altering the rate of Cofilin dephosphorylation. Conversely, depletion of Coronin 2A inhibits the formation of ATP-depletion induced Cofilin-GFP rods. These data indicate that Coronin 2A participates in the formation Cofilin-Actin rods and that ectopic expression of this protein induces the formation of rod-like structures that may be precursors to Actin-Cofilin rods.

985/B143
Functionally Distinct Actin Structures in Fission Yeast Are Generated by the Coordinated Action of Diverse Actin Filament Side-Binding Proteins.
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The assembly of actin structures with different functional properties requires specific combinations of actin-binding proteins. We are utilizing a combination of biochemical, biophysical, genetic and cell biology assays to examine how actin filament side-binding proteins contribute to the assembly of actin cables, actin patches, and the contractile ring in the fission yeast Schizosaccharomyces pombe. We found that tropomyosin SpTm (stabilizing), fimbrin Fim1 (crosslinking) and cofilin Adf1 (severing) competitively bind to and differentially influence actin filament architecture and function. SpTm is targeted to contractile ring actin filaments associated with formin Cdc12. SpTm subsequently increases the length of formin-nucleated filaments by enhancing the rate of elongation and end-to-end annealing, which ultimately contributes to formin inhibition. We also found that SpTm protects filaments from severing by cofilin, which is localized both to the contractile ring and to endocytic actin patches. In contrast to its effects on SpTm, formin appears to negatively regulate fimbrin by reducing crosslinking. Fim1 binds with equal affinity to ATP-, ADP-Pi-, and ADP-actin and is localized partially to the contractile ring and strongly to endocytic patches. We found that Fim1 forms both parallel and anti-parallel bundles, has little effect on severing by cofilin, but dissociates SpTm from actin filaments. Displacement of SpTm by Fim1 has multiple implications: (1) in vitro, removal of SpTm by fimbrin “deprotects” filaments, allowing severing by cofilin and (2) in vivo, fimbrin and SpTm are genetically antagonistic and fimbrin controls localization of SpTm by excluding SpTm from endocytic patches. Our findings suggest that the differential association of diverse actin-side binding proteins to particular actin structures has important functional consequences.

986/B144
Two Actin-Interacting Protein 1 Isoforms Have Overlapping and Essential Function in C. elegans Development and Reproduction.
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Actin-interacting protein 1 (AIP1) is a unique WD-repeat protein that contains two seven-bladed propellers in one molecule. AIP1 promotes disassembly of actin filaments in cooperation with ADF/cofilin. In multicellular organisms, multiple isoforms of ADF/cofilin with different functions are present, but how ADF/cofilin isoforms functionally interact with AIP1 is not clearly understood. In the nematode Caenorhabditis elegans, two ADF/cofilin isoforms, UNC-60A and UNC-60B, have different functions. Previously, we showed that UNC-78, an AIP1 protein, specifically cooperates with UNC-60B, but not with UNC-60A, to disassemble actin filaments and regulate actin organization in muscle. In addition, C. elegans has a second AIP1 gene, aipl-1 (AIP1-like protein 1) with unknown function. The sequence of AIP1-1 is 66% identical to that of UNC-78 and predicted to have two seven-bladed propellers. AIPL-1 was expressed in embryonic body wall muscle, intestine and somatic gonad. Recombinant AIPL-1 preferentially interacted with UNC-60B In Vitro in a similar manner to UNC-78. AIPL-1 enhanced disassembly of actin filaments in the presence of UNC-60B. In the absence of UNC-60B, AIPL-1 did not enhance disassembly. Importantly, AIPL-1, as well as UNC-78, did not enhance actin disassembly in the presence of
UNC-60A. Since UNC-78 and AIPL-1 are the only AIP1 proteins in *C. elegans*, these results suggest that UNC-60B is the only ADF/cofilin isoform that functionally interacts with AIP1. An *unc-78*-null mutant was homozygous viable and showed severe disorganization of actin filaments in striated muscle. An *aipl-1*-null mutant was homozygous viable and showed no detectable phenotypes in actin organization. However, depletion of both *unc-78* and *aipl-1* caused severely disorganized actin filaments in body wall muscle and resulted in lethality as late embryos. Depletion of *unc-78* and *aipl-1* in larval/adult stages resulted in sterile animals with disorganized actin filaments in the somatic gonad. These results indicate that the two *C. elegans* AIP1 isoforms have similar activities to regulate actin filament organization *In Vivo* and essential for development and reproduction in *C. elegans*.

987/B145
A Mechanism for Actin Stress Fiber Homeostasis through Rounds of Damage and Zyxin-Mediated Repair.
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Actin stress fibers (SF) are subjected to dynamic stress loads originating from both inside the cell, as from SF contraction, and from outside of the cell, as from mechanical changes in the extracellular matrix. While force imbalances may induce catastrophic breakage of SF, not all SF disruptions progress to full breaks. By imaging live cells expressing fluorescently tagged actin, we observed sites along actin SFs undergoing rapid cycles of localized elongation and thinning. Thinning and elongation were followed by cessation of elongation and restoration of actin, thereby avoiding progression to a full break. By imaging cells expressing fluorescent tagged SF proteins, we found that the LIM domain scaffolding protein, zyxin, accumulated rapidly along sites of SF thinning and elongation, as did the actin polymerization facilitator VASP, and the actin bundling protein α-actinin. Using micromanipulation, we show that local accumulation of zyxin on deformed SF could be induced by direct mechanical stimulation; and using traction force microscopy, we found that zyxin accumulation/local elongation events function to relieve mechanical stress. In cells generated from zyxin -/- mice, most SF thinning/elongation events were not associated with recruitment of either VASP or α-actinin, and actin failed to recover, resulting in a five-fold increase in SF breaks than their wild-type counterparts, illustrating a key role for zyxin in maintenance of SF integrity. To clarify the roles of α-actinin and VASP at SF thinning/elongation sites, we expressed in zyxin null cells zyxin constructs mutated to disrupt VASP or α-actinin binding. This revealed that both VASP and α-actinin accumulation at SF thinning/elongation sites is dependent on binding to zyxin. We found that both VASP and α-actinin function to stabilize the elongation site. α-Actinin, but not VASP, functions to restore actin. Thus, SF damage sites recruit a zyxin-dependent repair complex that serves as a homeostatic system for maintaining SF integrity in the face of mechanical stress.

988/B146
Analysis of Conformational Changes in Wiskott Aldrich Syndrome Protein (WASP) Using a Split YFP.
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The Wiskott Aldrich Syndrome (WAS) is an X-linked recessive disease caused by mutations in Wiskott Aldrich Syndrome Protein (WASP). To study how WASP functions, the Bi-molecular Fluorescence Complementation (BiFC) assay in *Saccharomyces cerevisiae* cells was utilised to examine the conformation of WASP. WASP adopts a closed conformation when the VCA domain interacts with the GTPase Binding Domain (GBD). In this assay, the YFP molecule is split into 2 fragments and fused to the two ends of WASP, generating YFP₁₋₁₅₄-WASP-YFP₁₅₅₋₂₃₈ (WASP sensor). When WASP adopts the closed conformation, S. cerevisiae cells expressing the WASP sensor will exhibit higher levels of fluorescence due to the complementation of the two fragments
of YFP. The results showed that in the presence of WASP interacting protein (WIP) or WIRE, S. cerevisiae cells expressing the WASP sensor exhibited enhanced levels of fluorescence. However, when the WASP sensor was expressed together with Toca-1 or Nck-1, the cells had reduced levels of fluorescence even in the presence of WIP. These suggest that WIP or WIRE stabilised the closed conformation of WASP while Toca-1 or Nck-1 disrupted this conformation. Mutations in the different domains of WASP can affect the severity of WAS, giving rise to X-linked thrombocytopenia and X-linked severe congenital neutropenia. Using the BiFC system, fluorescence analysis of cells expressing the WASP sensor mutated in the WH1, GBD and VCA domains suggest that these mutations relieved the closed conformation of WASP. This assay was also carried out using mammalian cells in which verprolins were transfected along with WASP sensor or WASP sensor mutants in 293T cells. Fluorescence analysis of these cells was carried out using FACS and the results obtained were similar to findings in the yeast cells. Together, these findings indicate that the conformation of WASP is important in the regulation of its functions. Mutations in the different domains may affect interactions with its cytoplasmic partners, causing changes in the conformation. This work was supported by Agency for Science, and Technology and Research (A*STAR), Biomedical Research Council grant A*STAR 05/1/22/19/392.

989/B147
Actin Binding Protein-1 Interacts with WIP to Regulate Growth Factor-Induced Dorsal Ruffle Formation.
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Growth factor stimulation induces the formation of dynamic actin structures known as dorsal ruffles. Mammalian actin binding protein-1 (mAbp1) is an actin binding protein that has been implicated in regulating clathrin-mediated endocytosis; however, a role for mAbp1 in regulating the dynamics of growth factor-induced actin-based structures has not been defined. We show that mAbp1 localizes to dorsal ruffles and is necessary for platelet-derived growth factor (PDGF)-mediated dorsal ruffle formation. Furthermore, we demonstrate that mAbp1 and the related F-actin binding protein cortactin have non-redundant functions in dorsal ruffle regulation. We show that mAbp1 is a calpain 2 substrate in fibroblasts and that the preferred cleavage site occurs between the actin binding domain and the proline rich region, generating a C-terminal mAbp1 fragment that inhibits dorsal ruffle formation. We also demonstrate a novel, direct interaction between mAbp1 and the actin regulatory protein Wasp Interacting Protein (WIP) that is abrogated by a mutation in the mAbp1 SH3 domain. We show that the interaction between mAbp1 and WIP is important in regulating dorsal ruffle formation and that WIP-mediated effects on dorsal ruffle formation require mAbp1. Together, these findings suggest a novel role for mAbp1 in dorsal ruffle formation through its interaction with WIP.

Actin Dynamics and Assembly I (990 – 1009)

990/B148
Tropomodulin3 Nucleates Actin Polymerization.
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Tropomodulins (Tmods 1-4) are a family of actin pointed end-capping proteins that are expressed in a wide variety of cells and tissues. We have shown previously that tropomodulin3 (Tmod3), a ubiquitous isoform of Tmods, maintains cell shape of cultured human intestinal epithelial Caco-2 cells (Weber, K.L., Fischer, R. S., and Fowler V.M. (2007) J. Cell Sci. 120, 3625-3632). Tmod3 localizes to lateral membranes in Caco-2 cells. Reduction of Tmod3 levels by shRNA leads to a loss of actin filaments from lateral cell membranes, and a decrease in cell height, suggesting that Tmod3 stabilizes actin filaments at lateral cell membranes. However, how Tmod3 regulates actin...
filaments in epithelial cells remains unclear. In this study, we show that mouse Tmod3 (mTmod3) nucleates actin polymerization in vitro. In a pyrenyl-actin fluorescence polymerization assay, mTmod3 accelerated polymerization of 4 μM actin at nanomolar amounts (25 nM-200 nM) in a dose-dependent manner. Gel filtration analysis and chemical cross-linking assay with 1-ethyl-3-(dimethylaminopropyl)carbodiimide/N-hydroxysulfosuccinimide (EDC) showed that mTmod3 formed a stable 1:1 complex with monomeric actin. Mutagenesis of Leucine73 to Aspartic acid, which is predicted to destroy the α-helix2 in mTmod3, significantly decreased both its monomer-binding and nucleating activities, indicating that mTmod3 binds to monomeric actin via its α-helix2, and that the monomer-binding activity of mTmod3 is necessary for its nucleating activity. These results suggest that Tmod3 may increase the amount of actin filaments by nucleating actin polymerization, and then stabilize filaments by capping their pointed-ends in epithelial cells.

991/B149
Nck Function in Tyrosine Kinase Signaling to the Actin Cytoskeleton.
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Tyrosine kinase signaling leads to the post-translational modification of proteins and their binding partners. These modifications lead to the membrane recruitment of signaling proteins, promoting an increase in their local concentration, which results in a cellular response to the phosphorylation of tyrosine residues. Nck, an SH2/SH3 adaptor protein, functions in tyrosine kinase signaling by linking tyrosine phosphorylation on the membrane with binding partners that function in facilitating actin nucleation and polymerization. However, quantitative and mechanistic aspects of signaling through Nck remain poorly understood. To explore the linkage of Nck to the actin cytoskeleton, our lab developed a system in which Nck SH3 domains can be aggregated on the plasma membrane following antibody application. Aggregation of Nck SH3 domains results in localized actin polymerization in the form of actin comet tails. Using the Virtual Cell, we have built a comprehensive, quantitative actin cycle model. With this model, we have produced predicted results that have been confirmed in vivo. This model predicts experimental comet tail length, actin distribution within the comet tail, and maximum actin concentration in the tail based on the number of molecules in the aggregate and the speed at which the aggregate is moving across the cell surface. The combination of modeling and precise experimental manipulation provides unique insights into the relationship between increased local concentration of Nck and resulting localized actin polymerization.

992/B150
Activation of the Wave Complex by Coincident Signals Controls Actin Assembly.
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A large signaling network regulates actin assembly in eukaryotic cells. While many of the key players in these actin regulatory pathways have now been identified, how numerous signals are integrated to produce complex cellular behaviors such as chemotaxis is still poorly understood. SCAR/WAVE proteins are at the core of regulatory pathways driving membrane protrusion. They link upstream signals to actin nucleation by activating the Arp2/3 complex and are found in large heteropentameric complexes whose role in the regulation of SCAR/WAVE function is presently unclear. Here we demonstrate that purified native WAVE1 and WAVE2 complexes are basally inactive and that previous reports of constitutive activity appear to be artifacts of In Vitro manipulation; thus, activation is necessary for WAVE complexes to execute their cellular functions. However, we find that purified native complexes cannot be activated by the small GTPase Rac or the adapter protein Nck as previously proposed. Instead, activation of the WAVE2 complex requires simultaneous interactions with prenylated Rac-GTP and acidic
phospholipids including but not limited to the signaling phosphoinositiide PI(3,4,5)P3. Furthermore we show that phosphorylation of the complex in an EGF-independent manner is required for activation by Rac and acidic phospholipids. Thus, while it has been shown that phosphorylation of WAVE1 is a negative regulator of activity, we now demonstrate that phosphorylation of the WAVE2 complex is a positive regulator. Together these signals promote full activation of the WAVE2 complex in a highly cooperative process on the membrane surface, possibly setting a high threshold for local actin polymerization. Activation most likely happens through allosteric rearrangements in the complex, as we observe no dissociation of the constituent subunits. Our results establish the WAVE complex as a key node capable of integrating coincident signals from Rac, acidic phospholipids and kinase pathways to promote localized actin nucleation. This signaling module is probably central to the regulation of cell motility in diverse processes such as embryonic development, immune response and cancer metastasis.

993/B151
The WAVE/SCAR Complex Regulates Nuclear Migration during Embryonic Development.
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Dynamic remodeling of the actin cytoskeleton plays an essential role in all organisms. Our laboratory has established that in C. elegans the actin nucleation cassette encoded by Rac1-WAVE/SCAR-Arp2/3, hereafter referred to as the GEX (GTPase/Enhancer of nucleation/actin nucleation eXecution) complex, is essential for embryonic cell migration and morphogenesis. However, cell migration requires dynamic interplay between filamentous actin (F-actin) and microtubules (MTs). within a migrating cell, the nucleus migrates along MTs and is repositioned on F-actin which requires distinct complexes composed of two novel nuclear envelope protein families, SUN and KASH domain proteins. To date, the role of actin nucleators in nuclear migration and positioning remains unknown. Using 4D movies we observed nuclear migration defects in various tissues at different developmental stages upon depletion of GEX molecules. Loss of GEX components leads to nuclear migration failure in intercalating embryonic epidermal cells and in the larval P cells which generate five classes of motor neurons. Accordingly, mutations in known nuclear migration proteins, including UNC-84 (SUN) and UNC-83 (KASH) lead to similar defects. However, in the one-cell zygote the female and male pronuclei undergo a stereotypical migration that allows them to meet and fuse, subsequently to form the one-cell embryo. Embryos depleted of WAVE/SCAR components or Arp2/3 components display a defective anterior migration of the male pronucleus in the one-cell zygote that is accompanied by altered non-muscle myosin-II dynamics and apparent changes in the MT cytoskeleton. In conclusion, these studies establish that Arp2/3-dependent actin nucleation is an essential component of nuclear migrations throughout development, and provide a novel system to examine the interaction of the MT and actin cytoskeletons.

994/B152
SCAR/WAVE as a Regulator of Cell Shape and Traction Forces during Amoeboid Migration.
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Cell motility originates from the complex spatiotemporal interaction of different biomolecules resulting in a periodic movement consisting of distinct phases (motility cycle). Actin filament dynamics are a major driving force of this movement and are partially controlled by the activation of Arp2/3 through the SCAR/WAVE complex. Our aim is to identify the way individual members of the complex affect the dynamic behavior of actin filaments by comparing wild type (wt) migrating Dictyostelium cells with cell lines carrying mutations in members of the complex (Scar, Pir121, Nap125). Time-lapse images of Dictyostelium migrating towards a chemoattractant source on elastic substrate were acquired. Individual cells were tracked over time and a phase average analysis of the traction stresses and cell shape was performed by splitting each cycle into four
stages using instantaneous cell length changes as condition. We find that all mutants exhibit enhanced pseudopodal activity. In pirA- cells, there is additionally increased localized activation of actin polymerization, consistent with the known function of Pir121 as negative regulator of Scar. Our study revealed that all cells implement a shape and force regulated cycle whose duration was longer in the mutants which also had lower velocity. The spatial distribution of traction forces of wild-type and scr- cells was similar but their magnitude was significantly reduced in scr-, while there were no significant differences in the magnitude of the forces between wt and pirA- cells, but the region of the peak stresses was shifted considerably towards the center of the mutant cells. A similar stress distribution was observed in the napA- cells although with lower magnitude. Taken together, these results indicate that the mechanical cycle of traction stresses is modified when actin polymerization is down-regulated, possibly due to a partial loss of spatial regulation of the stresses generated by the altered cell shapes observed for these mutants. Further experiments will be needed to clarify whether this is due to an altered F-actin architecture downstream of Arp2/3 or whether additional effectors of the SCAR/Wave complex are involved.

995/B153
Crosslinking Molecules Inhibit the Depolymerisation of Actin Filaments.
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The dynamic properties of the cytoskeletal polymer actin play a crucial role for the motility and adaptability of eukaryotic cells. The polymerization and depolymerization behaviour of filamentous actin solutions have been extensively studied in In Vitro experiments. However, the effect of cross-linking and bundling proteins, which determine the structural arrangement of actin in vivo, on the kinetics of actin is rather unknown. Utilizing multiple depolymerization assays we show that actin filaments are stabilized by cross-linking and bundling molecules. The bonds between cross-linking molecules and actin slow the depolymerization processes in a concentration dependent manner down, at one point depolymerisation is even totally inhibited on relevant time scales. This general effect can be rationalized by a simple model. The drastic modification of actin kinetics by cross-linking molecules can be expected to have wide-ranging implications for living cells, where cross-linking molecules are omnipresent.

996/B154
Contractile Ring Assembly in S. pombe as an Emergent Behavior of Filaments, Crosslinkers, and Motor Proteins.
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Complicated cellular behaviors emerge from networks of simple molecular and/or force-based interactions, and they underlie all of biology. Our failure to understand such “emergent phenomena” limits progress today in many areas of research. We therefore urgently need new methods for dealing with such complexity. One very promising tool is so-called agent-based computer modeling, which involves the explicit simulation of small-scale local interactions, following the trajectory through time of thousands to millions of states. The construction and application of these models integrates classical mechanics with biophysics and computer science, and it requires a great deal of data from experimental cell biology. Detailed agent-based simulations give us a way to explore, through a blizzard of arithmetic on fast memory-laden computers, the complex emergent behaviors that characterize all interesting cellular behaviors. This mimicking of biological systems in silico does not generate an elegant mathematical encapsulation of a system. but it has the great advantage of avoiding any need to intuit the outcome of myriad biochemical and force feedback loops, a task at which human intelligence is demonstrably frail. I present features of a 3-dimensional agent-based modeling framework, describe biophysically realistic actin filament and myosin motor representations. This model, focused on contractile ring assembly in fission yeast, demonstrates that the formation of a linear
contractile structure from an initial wide distribution of protein nodes is a robust emergent property of the interplay between filaments, crosslinkers, and motor proteins.

**997/B155**

**An Arg: Cortactin Complex Mediates Adhesion-Dependent Fibroblast Edge Protrusion and Breast Cancer Cell Invadopodia Function.**

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Abl family kinases are key regulators of actin-based cell edge protrusions during cell:matrix adhesion. The molecular mechanisms by which the Abl or Arg kinases interface with the actin polymerization machinery to promote these protrusions are unclear. We show here that interactions between Arg and the Arp2/3 complex regulator cortactin are essential to mediate actin-based cell edge protrusion during fibroblast adhesion to fibronectin. Arg-deficient and cortactin knockdown fibroblasts exhibit similar defects in adhesion-dependent cell edge protrusion, which can be restored via re-expression of Arg and cortactin. Arg interacts with cortactin via both binding and catalytic events. The cortactin Src Homology (SH) 3 domain binds to a proline-rich motif in the Arg C-terminus. Arg mediates adhesion-dependent phosphorylation of cortactin, creating an additional binding site for the Arg SH2 domain. Mutation of residues that mediate Arg:cortactin interactions abrogate the abilities of both proteins to support protrusions and the Nck adaptor which binds phospho-cortactin, is also required. Furthermore, using RNAi-mediated knockdown, rescue mutations, and biochemical studies, we show here, for the first time, that a similar pathway is involved in regulation of specialized matrix degrading protrusive structures in invasive breast cancer cells, called invadopodia. These results demonstrate that interactions between Arg, cortactin and Nck1 are critical to promote adhesion-dependent cell edge protrusions in both fibroblasts and invasive breast cancer cells.

**998/B156**

**Matrix Tension Regulates Trafficking of the Serum Response Factor Co-Activator Mal through Changes in Actin Dynamics.**

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Tensional homeostasis in tissues depends on the actin cytoskeleton but the mechanisms by which actin dynamics are used to sense tension and maintain homeostasis, and how this affects transcriptional regulation to induce changes in cell fate, are not known. The Serum Response Factor (SRF) co-activator MAL, whose nuclear trafficking and activity is regulated by the level of monomeric actin in cells, has been implicated in tension-based regulation of SRF-mediated transcriptional activity. We used fibroblast-populated collagen matrices to test the role of MAL in tissue contraction and mechanotransduction in 3D. Overexpression and shRNA-mediated down-regulation studies showed that MAL regulates cell-mediated matrix contraction. Furthermore, acute mechanical stress induced rapid nuclear accumulation of MAL in cells in monolayers, but not when the cells had reached tensional homeostasis in anchored gels. Serum- or drug-induced nuclear accumulation of MAL in free-floating low-tension gels (where cells haven’t reached tensional equilibrium) was similar to that seen in 2D monolayers. However, MAL nuclear accumulation was drastically reduced in cells in anchored gels, where the tension was higher and the cells reached tensional homeostasis. This was accompanied by a higher G/F actin ratio, increased cofilin expression and higher unphosphorylated/phosphorylated cofilin ratio, as well as defective MAL nuclear import compared to cells in relaxed gels. Taken together, our results
suggest that the MAL/SRF pathway underlies mechanotransduction and tensional homeostasis through the modulation of actin dynamics.

**999/B157**

**Assembly Dynamics of Contractile Actomyosin Networks Modulated by Extracellular Matrix Stiffness.**

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The ability of adherent cells to regulate traction forces on their extracellular matrix (ECM) is fundamental to tissue morphogenesis and directed cell migration. To a large degree, cellular traction forces are regulated by myosinII generated cell contractility, which promotes the development of contractile F-actin bundles, the growth of mechanosensitive focal adhesions (FA) and exertion of large traction stresses on the ECM. However, it is unclear how these processes are regulated in time and in response to environments of varied stiffness. To address how myosinII ATPase activity drives the organization of the F-actin cytoskeleton into structures capable of efficient force transmission, we utilized a combination of high resolution confocal microscopy and traction force microscopy. We found that treatment with and removal of blebbistatin, a myosinII ATPase inhibitor, induced an exponential recovery of stress fibers, focal adhesions, and traction stress. However, the rates of recovery are strongly dependent on the elastic modulus of the ECM; with near complete recovery of FA size and traction stress occurring on the order of 10min on stiff (2.8kPa) matrices and 1min on soft (0.6kPa) matrices. These times correlated with the appearance of a regular myosinII pattern, oriented along actin bundles that reorganized from a punctate and random orientation at the onset of blebbistatin washout. Furthermore, this recovery was inversely proportional to retrograde F-actin flow speed, which decreased from 3.85 to 0.25um/min on 2.8kPa gels and 5.85 to 0.45um/min on 0.6kPa gels. Accordingly, these relationships indicate that, as focal adhesions assemble, F-actin rapidly reorganizes into compact bundles at timescales consistent with increase in force underlying adhesions and that these timescales are altered by ECM mechanics. We propose that enzymatic activity of Myosin II promotes rapid reorganization of the F-actin cytoskeleton until myosin-II mediated tensile stresses within the cytoskeleton are balanced with traction stresses exerted on the ECM.

**1000/B158**

**Roles of Myosin II in Initiation of Focal Adhesion Assembly during Their Restoration after Blebbistatin Treatment.**

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In nonmuscle cells, contractile actin bundles (stress fibers, SFs) and their anchorage sites (focal adhesions, FAs) depend on each other for assembly. However, it remains unclear how this cycle begins. Nonmuscle Myosin II plays a key role in this process. It polymerizes into bipolar filaments and generates tension in SFs, which is required for maturation of nascent focal complexes (FXs) into FAs. In contrast, FXs are thought to be independent of Myosin II activity. To study roles of Myosin II in the assembly of SFs and FAs, we induced disassembly of these structures by blebbistatin (BS) that inhibits the motor activity of Myosin II, and investigated their recovery after BS washout by light and electron microscopy. BS treatment dramatically decreased the fraction of cytoskeleton-associated Myosin II and the amount of bipolar filaments in REF52 fibroblasts and disrupted large SFs and FAs. Although many FXs survived treatment with 50-75 μM BS, they were strongly inhibited by 100 μM BS, which severely impaired cell adhesion leading to retraction of cell edges, transformation of flat lamellipodia into ruffles, and eventually to cell detachment. Tiny actin bundles remained in cell lamella, but they were not associated with FXs. Phosphorylated Myosin II became concentrated at cell edges after BS treatment instead of being distributed all over the cell. The recovery from 100 μM BS began with fast (within 1 min) redistribution of phosphorylated Myosin II away from cell edges and formation of flat lamellipodia with FXs at their bases. No significant formation of actin bundles or bipolar myosin filaments was
observed at this stage, but they became apparent after ~5 min of recovery. Subsequent recovery consisted of gradual and coordinated maturation of FAs and SFs. Our data suggest that Myosin II is required for formation of FXs and therefore for cell adhesion and effective protrusion. We propose that Myosin II is activated by phosphorylation at the cell edges and initiates FXs there even before it visibly assembles into bipolar filaments. These FXs support the subsequent SF assembly, which occurs when Myosin II filaments orient and cross-link actin filaments into bundles. Supported by UPenn Research Foundation.

1001/B159
Role of Non-Muscle Myosin II in Regulation of Cell Adhesion.
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The regulation of cell adhesion to its surrounding extracellular matrix (ECM) is essential for cell migration and the stability of multicellular assemblies. For stabilization of cell adhesion, the actin cytoskeleton, focal adhesions and integrin/ECM binding contribute to efficient cell adhesion in the presence of external forces. However, the contribution and regulation of each are largely unknown. Mechanical forces applied to focal adhesions drive changes in focal adhesion signaling and morphology, which are thought to perturb the nature of force transmission at the focal adhesion. In the absence of external forces, this tension comes predominately from the action of Myosin II motors on the actin cytoskeleton. To examine the role of myosin-II tension in regulation of cell adhesion strength, we treated NIH3T3 fibroblasts with different pharmacological inhibitors of Myosin II activity (Y27693 and Blebbistatin). While both inhibitors showed similar focal adhesion phenotypes, the cell adhesion strength, as measured by a modified spinning disk assay, differed. Inhibition of Rho kinase with Y27693 diminished adhesion strength by 30%. By contrast, inhibition of Myosin II ATPase activity reduced adhesion strength by 60%. To examine the isoform dependence on adhesion strength, we reduced expression of Myosin IIA (NMIIA) and IIB (NMIIB) using shRNA. Similar to previous results, we find that reduction of NMIIA expression results in a dramatic reduction of focal adhesion size and abrogation of associated stress fibers. By contrast, shRNA of NMIIB results in small reductions in focal adhesion length and minimal changes in F-actin morphology. However, the adhesion strength of cells with reduced expression of either NMIIA or NMIIB is diminished by 70%. These results suggest that stabilization of the actin cytoskeleton by Myosin II may play an important role in regulation of cell adhesion.

1002/B160
Transcriptional Feedback Loops in Regulation of Cellular Adhesion and Tension.
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The ability of a cell to sense its physical surroundings results in short term changes in cytoskeletal structure and also long term changes in gene expression. Through these responses, cells can modify their microenvironment by perturbations to the surrounding extracellular matrix and secretion of soluble cues to surrounding cells. To explore how changes to the level of intracellular or extracellular tension potentiate long term transcriptional changes associated with cellular adhesion, tension and migration, we used QPCR to examine gene expression of human foreskin fibroblasts after incubation with pharmacological inhibitors of the actomyosin cytoskeleton or letting the cells adhere to substrates of differing stiffnesses. To decrease intracellular tension, we treated the cells with a Myosin II ATPase inhibitor, blebbistatin. After 24 hours, we found a decrease in mRNA levels for some cytoskeleton associated and focal adhesion proteins, including alpha actinin-1 (atn-1), myosin heavy chains (MyoIIA and MyoIIB), myosin regulatory light chains (Mrlc2 and Mrlc3) and talin. Other cytoskeletal and adhesion genes, such as ActR3, CapZa1, Coro3 and paxillin, showed no change in mRNA levels. To perturb the tension in the extracellular matrix, cells were plated on substrates of varying stiffness, 550 Pa, 8 kPa or 42 kPa. This resulted in some changes in gene expression, most notably there was an increase in MyoIIIB expression on the softest substrate. Atn-1, an actin-crosslinking protein, was of particular interest as it is likely responsive to serum response factor (SRF), a transcription factor
with cofactors affected by the G-actin:F-actin ratio. Preliminary data suggests that the polymerization state of actin strongly influences the expression of atn-1 and other cytoskeletal genes. Thus, long term transcriptional regulation of genes that regulate cellular adhesion and migration appears to be modified by regulation of myosin-II contractility and actin polymerization.

1003/B161
Adaptive Cellular Remodeling to Mechanical Stimulation.
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Cells have dynamic structures that adapt continuously to their micromechanical environment. The most interesting cellular behaviors are highly dynamic and occur in specific cell regions: focal adhesions (FA) and actin turnover, protein translocation, etc. Thus, particular cellular changes have their own time and distance scales that have to be matched by the instruments that are used to analyze them. By integrating in one instrument the atomic force microscope (AFM) with total internal reflection fluorescence and spinning-disk confocal microscopy, we were able to quantify proteins dynamics and cell signaling in real-time. The mechanical stimulation was induced by an AFM probe functionalized with fibronectin, which was set at a chosen xy-coordinate on the cell surface and moved upward in discrete steps. Following each controlled upward movement of the functionalized probe along z-axis, the cell responds to the mechanical stimulation. Thus, the AFM mechanical stimulation combined with simultaneous optical imaging experiments allowed us to: (i) acquire images that show real-time actin restructuring due to AFM mechanical stimulation; (ii) measure changes in the relative fluorescence intensity over time in local regions of interest showing vinculin and actin recruitment at basal cell membrane and (iii) analyze the specific cell response to external mechanical force. The results showed that the basal cell area was reduced 8.7±2.1% after AFM mechanical stimulation, but vinculin area increased 11.1±2.1% (n=5). Also, F-actin at basal cell area increased 16.9±1.9% and F-actin in whole cell volume increased 8.2±0.3% (n=4). By recruiting FA proteins and F-actin at the basal cell area, the cell reinforces its attachment to the substrate to better resist the applied force. These experiments showed a significant rearrangement of F-actin and FA due to the local mechanical cell stimulation at the apical cell membrane that induced changes into the cellular structure throughout the cell body. Integration of these techniques enables real-time optical imaging of 3D molecular dynamics at a time-scale of milliseconds while applying mechanical stimulation in the range of pico- to nano-Newton forces.

1004/B162
Contractility of Active Actin Networks Powered by Myosin Motors and Orchestrated by Crosslinkers.
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In the cell, Myosin II motors are able to slide filaments of actin (F-actin) past one another, while actin crosslinkers are crucial for the build up of sustained tensions. These tensions are a common denominator in contractile processes like cytokinesis or cell locomotion. It is unclear, however, how the interplay between motor activity and crosslinking tunes contractility. To elucidate the effect of myosin motor activity and crosslinker density on the structure and dynamics of the actin cytoskeleton, we reconstitute an In Vitro minimal model system composed of F-actin, skeletal Myosin II and irreversible crosslinkers (biotin-streptavidin). We track microspheres in actin networks assembled in a flow cell and find that sphere probes are more mobile than in the networks without motors. These movements and flow-like group translocations of the probes indirectly reveal the contractile fluctuations of the actin mesh at different length scales. However, as the crosslinker density is increased above a threshold concentration, the network contracts as a whole, which leads to a macroscopic coordinated motion of the probes. Moreover, contraction
rates increase with crosslinker concentration. To image network rearrangements due to motor activity and crosslinking, we label the actin fluorescently and use confocal microscopy. We find that in loosely crosslinked networks motor activity induces the formation of ring-like structures. These assemblies are only present if myosin is sufficiently processive, i.e. if motors are assembled into thick filaments or minifilaments. However, unlike previously reported, it appears that crosslinking is not needed for such patterns to appear. These findings confirm that crosslinkers are necessary to support and enhance macroscopic force propagation in actin networks. on the other hand, myosin motors alone seem to be sufficient for locally remodeling the network.

1005/B163
Parameterization of the Actin Dendritic Nucleation Pathway: A Rate-Limiting Step.
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Dendritic nucleation of filamentous actin has been studied extensively, yet some important issues, such as a rate-limiting step, remain controversial. We present a computational analysis of recent experimental data showing that even for a relatively simple network of non-linear interactions, determination of a rate-limiting step can be a nontrivial task. First, the rate-limiting step may depend on experimental conditions and can even change in the process. Second, we have shown that the data typically measured in In Vitro actin polymerization assays, albeit extensive, are insufficient for unique parameterization of the reaction network. The analysis points to additional experiments with the In Vitro assays that are needed for unambiguous identification of the rate-limiting step in the pathway of actin dendritic nucleation. The work is supported by National Institutes of Health through grants 1U54 -RR022232, P41-RR13186, and 1U54-GM64346-01.

1006/B164
Spatiotemporal Organization of Actin Binding Proteins during Actin-Based Motility.
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Actin-based propulsion of pathogens and/or of functionalized objects allowed to reconstitute In Vitro the minimum motility medium made of purified proteins. The analysis of the mechanism of force production was widely achieved for all global parameters (protein concentration, object diameters, viscosity of the medium...) and leads to different models. Yet, how the different proteins necessary to sustain actin-based force generation (the Arp2/3 complex, ADF/cofilin and capping protein) are coordinated in space and time during motility is an open question. We used fluorescently labeled proteins and FRAP experiments to follow, in real time, the spatiotemporal distribution of these proteins during the motility of nucleation promoting factor (NPF)-coated beads. We find that unlike capping protein that co-localizes homogenously within actin comet tail, ADF/cofilin binding increases gradually away from the beads concomitant with the state of the nucleotide associated to the actin subunits. Our results show that the spatial localization of nucleation, elongation and ageing zones depends on the balance between local sustained nucleation of actin filaments by the Arp2/3 complex and capping protein activity. Moreover, we determined the efficiency of fragmentation by ADF/cofilin during motility at about one fragmentation event every 100 ADF/cofilin molecules present in the medium.

1007/B165
Waves and Patches in Dendritic Actin Nucleation.
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The polymerization dynamics of actin are crucial for cell migration and extension of protrusions. Recent fluorescence imaging studies have shown that F-actin can spontaneously form traveling waves or moving patches at low actin concentrations. I investigate possible mechanisms for such
phenomena by numerically simulating the "dendritic nucleation" model of actin network growth on a 3 by 3 micron piece of membrane. The simulations store information about actin filaments subunit by subunit, giving an explicit three-dimensional picture of the actin network. They include filament growth, capping, branching, severing, and random thermal motion implemented via Brownian dynamics. The dynamics of nucleation-promoting factors (NPFs) in the membrane are also included. They diffuse in the membrane, and detach in the presence of F-actin, thereby becoming inactivated; activation occurs by reattachment. The simulations show three types of dynamic F-actin behavior: 1) traveling waves, 2) coherently moving patches, and 3) random fluctuations with occasional moving patches. Wave formation at low G-actin concentrations is favored by a long recovery time for NPFs which have been inactivated, and by weakness of the attractive interaction between filaments and the membrane. Raising the G-actin concentration results in a randomly varying distribution of F-actin. Lowering of the G-actin concentration below the optimal value for waves causes the waves to break up into patches which, however, move coherently. Similar effects are seen with decreasing and increasing capping-protein concentration, respectively. Diffusion of NPFs slows the waves, and fast diffusion stops them completely, resulting in the formation of static spots.

1008/B166
A Perinuclear Actin Cap Controls Nuclear Shape.
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Defects in nuclear morphology often correlate with the onset of disease, including cancer, progeria, cardiomyopathy, and muscular dystrophy; defects in nuclear shape are routinely used in the lab and in clinical setting as markers of disease and differentiation in human cells and tissues. In 1921, Champy and Carleton suggested an apparent correlation between the shape of various types of animal cells and the shape of their respective nuclei. However, the mechanisms through which a cell controls its nuclear shape remain unknown. Here, using adhesive micropatterned substrates to control cell shape, we show that an actin filament structure forms a cap or dome above the apical surface of the nucleus tightly controls nuclear shape, as well as identify key associated cytoskeletal regulators of its organization and nuclear shape-determining function. The organization of this actin cap and its nuclear shape-determining function are disrupted in cells from mouse models of accelerated aging and muscular dystrophy with distorted nuclei caused by alterations of A-type lamins. We are also investigating the role of this actin cap in the differentiation and development of human embryonic stem cells.

1009/B167
Novel Mechanism of Actin Bundle Formation by Dynamin/Cortactin Ring Complex.
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Actin cytoskeleton is dynamically remodeled upon variety of cellular events including cell motility, adhesion, endocytosis, and phagocytosis. An endocytic GTPase dynamin 2 binds to cortactin, an actin filament binding protein, suggesting an implication of these proteins for actin dynamics. However, mode of action of dynamin and cortactin in the actin remodeling remains largely unknown. In this study, we demonstrate that cortactin and dynamin 1 co-assemble into rings, which directly bundle actin filaments. Interaction between dynamin 1 and cortactin was detected by GST-pull down assay. The interaction was mediated by cortactin SH3 domain and dynamin 1 proline rich domain. Incubation of preformed F-actins with dynamin 1 and cortactin in presence of GTP resulted in the assembly of F-actins into long and thick bundles. Quantitatively, actin bundle formation was markedly increased in the presence of both dynamin 1 and cortactin as assessed by low speed sedimentation assay. Dynamin 1 and cortactin were present at puncta periodically
present along the bundles by immunofluorescence, and EM analysis revealed that these dots represent clusters of dynamin/cortactin complexes bundling actin filaments. In the protein clusters, structures twining around several actin filaments were occasionally observed. Dynamin and cortactin WT, but not cortactin SH3 domain mutant, assembled into rings in the presence of GTP or GDP, or in the absence of guanine nucleotides. Dynamin 1/cortactin complexes were linear shape in the presence of GTPγS, suggesting that the configuration of the complex are changed upon GTP hydrolysis. Periodic presence of dynamin 1 and cortactin along actin bundles was observed on filopodia of neuronal growth cones. Similar localization was observed for dynamin 2 and cortactin on filopodia of non-neuronal cells. RNAi of dynamin or cortactin suppressed the filopodium formation. These results strongly suggest a novel mechanism through which dynamin/cortactin complexes directly bundle actin filaments.

Unconventional Myosin (1010 – 1023)

1010/B168
Localization of Myosin 1B to Actin Protrusions Requires Phosphoinositide Binding.

The class I myosin, Myosin 1b (Myo1b), is a widely-expressed, single-headed, actin-associated molecular motor. Transient kinetic and single-molecule studies indicate that it is kinetically slow and responds to tension. Although its role is unknown, localization and subcellular fractionation studies indicate that Myo1b associates with plasma membrane and certain subcellular organelles such as endosomes and lysosomes. Whether Myo1b directly associates with membranes is unknown. In this study, we demonstrate by pull-down assays with lipid-coated beads that full-length Myo1b binds specifically to phosphatidylinositol-4,5-bisphosphate (PIP$_2$) and phosphatidylinositol-3,4,5-triphosphate (PIP$_3$), two phosphoinositides that play important roles in cell signaling. We also show that binding is not Ca$^{2+}$-dependent, does not involve the calmodulin-binding IQ region in the neck domain of Myo1b, and that the binding site is contained entirely within the C-terminal tail region, which contains a putative PH domain within the tail homology 1 (TH1) domain. Single mutations in the putative PH domain abolish binding of the tail domain of Myo1b to PIP$_2$ and PIP$_3$ in vitro. The mutations alter the distribution of myc-tagged Myo1b at membrane protrusions in COS-7 cells where PIP$_2$ localizes. These results suggest that binding of Myo1b to phosphoinositides plays an important role in vivo.

1011/B169
Analysis of the Role of Myosin 1E in Podocyte Functions.
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Mammalian myosin 1e is a single-headed, actin-dependent molecular motor that contains lipid- and protein-interacting domains in its tail. A previous study has demonstrated that myosin 1e is expressed in glomerular epithelial cells (podocytes) and that knockout mice lacking myosin 1e develop severe kidney disease (Krendel, Kim, Willinger, Wang, Kashgarian, Flavell, and Mooseker, JASN, 2009, 20:86-94). Myosin 1e-null mice exhibit glomerular filtration defects and abnormal podocyte morphology. The goal of the present study is to identify myosin 1e binding partners in podocytes and to determine the role of myosin 1e in podocyte functions. Using In Vitro binding assays and co-localization analysis, we have found that the SH3 domain of myosin 1e tail interacts with two proteins known to be important for podocyte functions, synaptopodin and dynamin. Synaptopodin is a key regulator of cytoskeletal organization and cell signaling in podocytes while dynamin contributes to modulation of actin organization in podocytes as well as plays an important role in endocytosis in a variety of cell types. Based on the observed protein-protein interactions of myosin 1e tail, we propose that myosin 1e may be involved in regulation of cytoskeletal dynamics, cell motility, and/or endocytosis in podocytes. In order to further
investigate these possibilities, we have developed an adenovirus-based system for shRNA-mediated knockdown of myosin 1e in conditionally immortalized podocytes. The knockdown approach results in more than 90% decrease in the level of myosin 1e protein and provides an efficient experimental system for testing the proposed roles of myosin 1e in kidney functions.

1012/B170
Role of Myosin 1G in Plasma Membrane Dynamics.
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A dynamic plasma membrane is central to many physiological processes such as cell migration, membrane traffic and signaling. The dynamics of the plasma membrane is in part regulated by the actin cytoskeleton. Different actin binding proteins regulate actin dynamics in cells and myosin motor proteins are one such class of actin regulators. Class I myosin’s of the myosin superfamily are implicated in diverse functions such as hearing, membrane traffic, vesicular transport and force sensing. Class I myosin’s are single headed, non-processive motors with a polybasic tail region that binds phospholipids. We analyzed a previously characterized class I myosin, Myo 1G. Myo 1G is specifically expressed in hematopoietic tissues and cells. In cells Myo 1G localizes to the plasma membrane. Point mutations that abolish motor activity of Myo1G do not affect its localization to the plasma membrane. The Myo 1G tail has a putative PH-like domain that is essential, but not sufficient for localization to the plasma membrane. Full length Myo1G binds In Vitro specifically to mono phosphoinositide lipids, namely PtdIns(3)P, PtdIns(4)P and PtdIns(5)P. However, the mutations in the ‘PH-like’ domain that abolish plasma membrane localization in vivo, do not abrogate binding to mono-phosphoinositides in vitro. Jurkat T-cells depleted of Myo 1G by siRNA have a reduced cell elasticity as determined by atomic force microscopy. These changes in cell elasticity do not affect FITC dextran uptake. These results are suggestive of a role for Myo 1G in plasma membrane dynamics.

1013/B171
Regulation of Fission Yeast Myosin-I Motor Activity and Recruitment to Endocytic Actin Patches by a Calmodulin-Like Light Chain and Tropomyosin.
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Fission yeast myosin-I (Myo1p) localizes transiently to cortical actin patches where it participates in force production that helps ensure internalization of endocytic vesicles. In this study we used genetics, time-lapse cell imaging, and biochemical experiments to assess the role of a novel myosin light chain (Cam2p) and an actin-based regulator (tropomyosin) in Myo1p function. Cam2p binds Myo1p directly, providing a second light chain (in addition to calmodulin). Like myo1Δ mutants, cam2Δ cells are temperature-sensitive, exhibiting a loss of cell polarity and lethality at elevated temperatures. The morphological defects of a myo1Δ strain are not exacerbated in a myo1Δ cam2Δ double mutant, suggesting that Cam2p’s primary role lies with Myo1p. The lifetime and levels of Myo1p recruited to patches are significantly lower in a cam2Δ mutant. Cam2p co-localizes with Myo1p at patches, but surprisingly does not rely on Myo1p for localization. In vitro, loss of Cam2p has no effect on Myo1p’s ATPase activity or actin affinity, but reduces its ability to drive actin filament gliding by ~3-fold in motility assays. We were interested to test whether fission yeast tropomyosin (Cdc8p) influenced Myo1p function, given Cdc8p localizes at actin cables, but not actin patches. In vivo, Myo1p localization was normal in a cdc8 mutant, although its rate of accumulation at patches was faster. In vitro, Cdc8p completely inhibited the ATPase and motility activities of Myo1p by blocking actomyosin interactions. Overall our data suggests that Cam2p and Cdc8p participate in the spatial regulation of Myo1p. Cam2p promotes the recruitment and activity of Myo1p at actin patches, whereas tropomyosin prevents Myo1p from binding actin cables, restricting its function to the actin patches.
1014/B172

Secretory Vesicle Transport in Yeast Requires the Direct Binding of Myosin V to Multiple Rab GTPases.

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Myosin V motors move multiple cargoes to distinct places at different times. This raises the question of how the distribution of these cargoes to their proper locations is achieved. Specificity is determined in part through the regulation of myosin V attachment to organelle specific receptors. At least five organelle-specific receptors interact with the cargo-binding domain of a yeast myosin V, Myo2. Based on the high-resolution structure of the Myo2 cargo-binding domain, and site directed mutagenesis, a surface area of Myo2 required for secretory vesicle movement was identified. This region binds directly to the Rab GTPases Ypt31 and Ypt32. Ypt31/32 function in polarized secretion and are required both for the formation of post-Golgi transport intermediates, and for their attachment to Myo2. Here we show that the Rab-GTPase Sec4, which functions downstream of Ypt31/32, also binds directly to the cargo-binding domain of Myo2, at the same site utilized by Ypt31/32. Sec4 is required for fusion of secretory vesicles with the plasma membrane. A point mutation in the center of the Rab binding site, myo2-Y1415R, is lethal for yeast growth. However, expression of fusion proteins of either Myo2-Y1415R-Ypt32 or Myo2-Y1415R-Sec4 as the sole copy of Myo2 suppresses the lethal phenotype. Notably, growth of the strain expressing the Myo2-Y1415R-Sec4 fusion is significantly better than growth of the strain expressing Myo2-Y1415R-Ypt32. These observations suggest that direct binding of Sec4 to Myo2 occurs subsequent to Ypt31/32 interaction with Myo2. The direct involvement of each of these Rab GTPases in the attachment of secretory vesicles to Myo2 suggests a mechanism that couples vesicle formation, movement and subsequent fusion.

1015/B173

Myosin Va Pulls the Endoplasmic Reticulum into the Dendritic Spines of Purkinje Neurons.

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Animals with mutations in dilute (MYO5A), the gene encoding the class V myosin Myosin Va, lack ER within the dendritic spines of their cerebellar Purkinje neurons (PNs) (Takagishi et al., Neurosci Lett 215, 169-72). One consequence of this defect is that cerebellar long-term depression, a form of synaptic plasticity underlying motor learning, is disrupted in dilute animals (Miyata et al., Neuron 28, 233-44). To investigate how Myosin Va drives the localization of ER into PN spines, we developed an efficient method of expressing cDNAs in PNs present within dissociated cerebellar cultures. Live cell imaging of PNs cultured for 15 days In Vitro (DIV) and simultaneously expressing an mRFP-tagged luminal ER marker and GFP as a cell volume marker confirms that ER is present in essentially all wild type (WT) PN spines, while it is completely absent from dilute (d20j/d20j) PN spines. Interestingly, whereas the ER is continuously present in WT spines at 15 DIV, we observed movements of ER into spine-like protrusions initially devoid of this organelle at 8-10 DIV. This ER movement is abolished in dilute PNs. Rescue experiments, where WT or mutant versions of Myosin Va are specifically expressed in dilute PNs, show that the myosin functions cell autonomously within PNs to mediate ER targeting, and that the myosin’s ability to hydrolyze ATP is required for ER insertion into spines. Moreover, Myosin Va accumulates at the leading tip of the ER tubule as it moves into the spine, consistent with the myosin pulling the ER. Finally, attenuation of the myosin’s ability to move along actin filaments by shortening its lever arm or slowing its ATPase activity using a switch 1 mutation both reduce the velocity of ER movement into spines, providing direct proof that Myosin Va drives ER motility. Thus, while it has been suggested that animal class V myosins localize organelles by dynamically tethering them to the actin cytoskeleton, we demonstrate here that Myosin Va is a point-to-point organelle transporter that translocates ER as a cargo into PN spines.
1016/B174

**In Vivo Analysis of Myosin Xi Function in Polarized Plant Cell Growth.**

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Myosin XIs are plant-specific and most similar to myosin Vs from animals and fungi. In plants, myosin XIs are responsible for cytoplasmic streaming, but their role in polarized growth is not well understood. Because of the large number of myosin XI genes in angiosperms, it has been difficult to determine their precise role. In contrast, in the moss *Physcomitrella patens*, there are only two myosin XI genes, which encode proteins that are 94% identical. To determine the role of myosin XI in polarized growth, we simultaneously silenced the expression of both myosin XIs by RNA interference (RNAi). Loss of myosin XI function results in a dramatic loss of polarized growth; plants are stunted and composed of small rounded cells. Interestingly, this phenotype is very similar to that caused by silencing proteins involved in regulating actin dynamics, suggesting that myosin XI may regulate actin turnover. We have also determined that the two myosin XI genes are functionally redundant by using specific RNAi constructs from the 5′ untranslated regions. These constructs do not produce a phenotype, demonstrating that a single copy of myosin XI is sufficient for polarized growth. Importantly, expression of a construct generated by combining the 5′ untranslated regions from each myosin XI phenocopies the coding sequence construct. In addition, this construct enables transient complementation studies, which are critical for the elucidation of the mechanism of myosin function in cell. Using this complementation assay, we show that GFP-myosin XIa completely rescues the myosin XI RNAi phenotype and localizes to the tip of the growing cell. To further dissect myosin XI mechanism of action during polarized growth, we are also analyzing the specific effect of myosin XI loss-of-function in intracellular motility.

1017/B175

**Caenorhabditis elegans Myosin IX Is a Plus-End-Directed Motor.**

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Mammalian myosin IXb is a single-headed motor that moves processively along actin filaments. The directionality of its movement along actin filaments is currently a matter of debate. In one report native myosin IXb moved towards the plus-end, whereas in another report truncated recombinant myosin IXb moved towards the minus-end. To resolve this discrepancy and to further characterize the motor properties of class IX myosins, we set out to study myosin IX (Myo9) from *Caenorhabditis elegans*. We generated constructs containing either the head domain (Myo9-head) or the head domain and the light chain binding region (Myo9-4IQ). The constructs were expressed and purified from SF9 cells. Steady-state F-actin-activated ATPase measurements performed at 20 °C revealed a vmax of 2.4 ± 0.1 s⁻¹ for Myo9-head and a ten-fold lower vmax of 0.23 ± 0.03 s⁻¹ for Myo9-4IQ. Specific adsorption of the constructs to a surface via biotin-streptavidin resulted in actin filament gliding. The F-actin gliding velocity of Myo9-head and Myo9-4IQ was determined to be 56.9 ± 0.7 nm s⁻¹ and 109.5 ± 8.2 nm s⁻¹, respectively. The velocity of actin filament gliding did not change with different Myo9-head densities. At low Myo9-head densities nodal pivoting of moving filaments was observed. Using polarity marked filaments, we observed that both Myo9-head and Myo9-4IQ moved towards the plus-end of actin filaments. We conclude that class IX myosins are plus-end directed motors.

1018/B176

**Is Myosin-X Required for Formation of Microvilli?**

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Myosin-X (Myo10) is an unconventional myosin that localizes to the tips of filopodia and is required for the formation of filopodia (Bohil et al. PNAS 2006). Microvilli share some similarities with filopodia, but differ in that they are found at the apical domain of polarized epithelial cells and...
are involved in absorptive and secretory functions. Given that both microvilli and filopodia are slender cellular extensions based on actin bundles, we tested whether myosin-X plays a role in the formation of microvilli using Caco-2 cells, a well-characterized model for microvilli. Western blotting demonstrated that myosin-X is present in Caco-2 cells at all time points tested, from spreading (1 day) to fully polarized cells (14-19 days). Myosin-X expression increases during early stages of Caco-2 development, peaks at 6 days, and then decreases to lower levels. Immunolocalization showed that myosin-X localizes to the tips of filopodia-like projections in spreading Caco-2 cells (1-3 days). In fully polarized Caco-2 cells, however, myosin-X is found in diffuse cytoplasmic puncta but is not detected in microvilli. To test whether myosin-X function is required for microvillar formation, stable myosin-X knock down cells generated by a lentiviral shRNA expression system were plated and grown to maturity (2 weeks). Scanning electron microscopy of the apical surface showed a reduction in the number of microvilli in Myo10 shRNA infected cells compared to non-specific shRNA infected cells. Interestingly, some Myo10 knock down cells exhibited prominent membrane ruffles at the apical surface of fully polarized cells. Expression of the Myo10 coiled-coil, a putative dominant negative, also led to a reduction in microvilli and presence of membrane ruffles. These results indicate that myosin-X is necessary for the formation of apical microvilli and suggest that filopodia may be required for the formation of microvilli. (Supported by NIH/NIDCD grant DC03299 to REC)

1019/B177
Myosin-XIX Is an Unconventional Myosin Involved in Mitochondrial Dynamics.
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Myosin-XIX (Myo19) is the founding member of a novel class of unconventional myosin. Bioinformatic analysis indicates that Myo19 orthologs can be found in chordates, and that Myo19 is broadly expressed in human tissues. Both immunostaining and expression of GFP-Myo19 truncations show that Myo19 localizes to mitochondria. Fluorescence recovery after photobleaching (FRAP) of GFP-Myo19 tail indicates that exchange with other cellular pools is slow, requiring several minutes to recover. Ectopic expression of full length GFP-Myo19 results in a gain-of-function phenotype where individual mitochondria can be observed to move for many microns with one end leading. To determine if the mitochondrial network as a whole was more dynamic, we calculated a Displacement Index (D.I.) by dividing the area taken up by mitochondria in the first frame of a time-lapse by the area which had been populated by mitochondria over the course of the entire time-lapse. A D.I. of 1 indicates that the mitochondrial network was perfectly stationary, and D.I. greater than one indicate a more dynamic mitochondrial population. for A549 cells expressing GFP-Myo19, the D.I. increased by 75% over control cells (2.1±0.7 versus 1.2±0.1). Additionally, GFP-Myo19 expression often results in “tadpole” shaped mitochondria, where the leading end of the organelle is wider than the trailing end. Neither coexpression of GFP-Myo19 with mRFP-utrophin nor correlative time-lapse microscopy reveals Listeria-like f-actin tails behind moving mitochondria, indicating that mitochondrial motility due to polymerization-mediated pushing is unlikely. Cells expressing GFP-Myo19 constructs containing mutations hypothesized to interfere with normal myosin function fail to induce either an increased D.I. of the mitochondrial network, or long range, end-on movements of individual mitochondria. Additionally, a purified construct consisting of the motor domain and neck of Myo19 displays actin-activated ATPase activity. Taken together, these data suggest that Myo19 is an unconventional myosin involved in mitochondrial movements. Supported by a NIH/NIDCD DC03299 to REC, a SPIRE postdoctoral training grant NIH/GM00678 to OAQ, and MBL Summer Research Fellowship to OAQ.
Myosins constitute a large superfamily of proteins with diverse properties adapted for a variety of important cellular activities. The relevance of myosins for mammalian physiology and pathology is underscored by the finding that many pathological conditions and genetic diseases are associated with mutations in myosins. Some important questions that remain unanswered in the field of myosin pertain to the role of the domains present in a particular region of the proteins called the tail. Two of these domains, the MyTH4 and the FERM are often found coupled in myosins of different classes but so far not much known why these two domains are coupled or what the individual role of each of the domains is. To investigate these two domains in myosin we have chosen Myo1, which is one of 13 myosins in *Tetrahymena thermophila* and the founding myosin in ciliates. The tail region of Myo1 is known to have a MyTH4 and a FERM domain but the functional role of either remains elusive. This present work comprises the sequence and structure analysis of these two domains to correctly identify their domain boundaries and to model the biophysical properties of their sequences onto their computationally modeled three-dimensional structures. Our results help to predict biological function for these domains and provide a starting point for understanding why mutations in these domains in different myosins and other proteins result in pathogenic conditions.

Tetrahymena contains two distinct nuclei, a micronucleus and a macronucleus. The micronucleus divides mitotically and is transcriptionally silent during vegetative growth but active during conjugation. The macronucleus divides amitotically and is transcriptionally active throughout the cell cycle and early in conjugation. Late in conjugation the macronucleus disintegrates, and a new macronucleus is formed from a zygotic micronucleus. Intranuclear microtubules assemble during amitosis. However, the precise role of these microtubules is unknown. Previous studies show that in a genomic knockout of MYO1 (myosin class XIV), elongation of the macronucleus, which normally precedes division of the nucleus, often fails to occur, and the ensuing division of the macronucleus is grossly unequal. Developmentally-regulated destruction of the macronucleus fails to occur during conjugation of the knockout. The present study aimed to identify a Myo1 domain that could interact with intranuclear tubulin and actin and affect amitosis. The project focused on the Myo1 tail domain, which contains MyTH4, a potential site for interaction with tubulin and actin. Alignment of Myo1 MyTH4 with a talin actin-binding sequence, a MAP-2 tubulin-binding sequence, and the SV40 nuclear localization sequence (NLS) revealed putative actin-and tubulin-binding sequences, and an NLS-like sequence. GFP-MyTH4 localized in a diffuse pattern throughout the nucleoplasm of macronuclei and micronuclei in situ and in vitro. Cells that over-expressed GFP-MyTH4 contained multiple macronuclei, macronuclear fragments, and multiple micronuclei. Actin antibody co-precipitated GFP-MyTH4 fusion and tubulin from lysate of cells expressing GFP-MyTH4. Co-sedimentation assays performed with either whole cell extracts or anti-actin immunoprecipitation pellets revealed that F-actin (independent of ATP) and microtubules co-sedimented with GFP-MyTH4. We conclude that the Myo1 MyTH4 domain interacts with tubulin and actin, localizes to macronuclei and micronuclei, and affects macronuclear amitosis. [Supported by NSF]
Ser/Thr protein phosphatase 1 holoenzymes consists of a conserved catalytic subunit (PP1c) and diverse regulatory subunits that define substrate specificity, sub-cellular localization and activity of PP1c. Based on domain and primary amino acid sequence homology, TIMAP is a PP1 regulatory subunit in the myosin phosphatase (MYPT) family. Its expression is restricted to EC. MYPTs control PP1c activity toward myosin light chains (MLC), controlling their phosphorylation and consequently myosin-dependent cell motility. We previously reported that TIMAP regulates EC filopodia extension (JBC 282:25960, 2007). Here, we determined whether TIMAP controls PP1c activity toward the non-muscle MLC2 in EC. Endogenous TIMAP and MLC2 co-immunoprecipitated from EC lysates, and from Cos-7 cells when HA-TIMAP and flag-tagged MLC2 were overexpressed. However, In Vitro transcribed and translated MLC2 did not interact directly with TIMAP. Two distinct TIMAP siRNAs, but not scrambled siRNA consistently reduced TIMAP expression in human umbilical vein EC (HUVEC) by 75-90%. MLC2 Thr18/Ser19 phosphorylation was stimulated 4.03±1.79 fold by 1U/ml thrombin in control HUVEC, an effect that was completely inhibited by Rho kinase inhibitor Y27632. Thrombin-stimulated MLC2 phosphorylation was unaffected by siRNA mediated TIMAP knockdown (4.67±1.72 fold stimulation, mean ± SD, n=3exps). Similarly, the rate of MLC2 dephosphorylation, determined by blocking Rho kinase with Y27632, was not different between bovine aortic EC stably overexpressing dominant negative TIMAPP1c-, which cannot bind PP1c, and those expressing wild-type TIMAP. Hence, although TIMAP is a member of the MYPT family of PP1c regulatory subunits, it does not control PP1c phosphatase activity toward MLC2 in EC.

1023/B181
Proteomic Analysis of the Kinesin and Myosin Protein Superfamilies.
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The structurally related myosin and kinesin motor protein superfamilies are encoded by approximately 80 genes and dedicated to actin and microtubule-based work, respectively. Although several of these cytoskeletal motors are extensively characterized, the regulation and function of most remains unclear. We established a set of Hela cells lines stably transfected with recombinantly-tagged bacterial artificial chromosome (BAC) transgenes expressing recombinantly LAP-tagged (a EGFP-containing affinity tag) motor proteins to interrogate their function by microscopy, immunoprecipitation (IP), and mass spectrometry of the immune pellets. Out of the resulting 56 transgenic cell pools, the expected transgene was recovered from 39 by IP and 27 displayed specific sub-cellular localization patterns by live-cell video microscopy. Cross-comparison of IPs from a matrix of transgene and three distinct mitotic synchronization protocols permitted background subtraction to identify novel, high-confidence interaction partners that we validated with tagged transgenes and functional RNAi analysis. In a pilot analysis, we found that 10, of 12 motor proteins required for mitosis, are properly targeted as transgenes in cells and recovered both published and novel transgene- and mitosis-specific interaction partners. For instance, we found that KIF10 (Cenp-E) binds the microtubule plus-end stabilizing proteins CLASP1 and 2 in a protein kinase-dependent manner and is required for targeting these proteins in mitosis. We also identified novel interaction partners for proper targeting of KINESIN-13s and KINESIN-6 to centrosomes and the spindle midzone, respectively. Several non-conventional myosins and non-mitotic kinesins also recovered novel interactors relevant to intracellular transport, cell polarity, and mitochondrial dynamics. The resulting protein-interaction network suggests that motor proteins define distinct sub-cellular compartments during interphase, while several motors physically interact during mitosis. Finally, the set of novel motor interacting proteins provides a resource to extend our understanding of how motor protein activity is regulated coordinated in cells.
**Cytoskeleton Membrane Interactions (1024 – 1048)**

1024/B182

**A Laminin Dependent Switch in the Cytoskeletal and Exocytic Machinery Driving Neuritogenesis.**

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Neuritogenesis, a crucial step in nervous system development, requires coordinated cytoskeletal dynamics and addition of lipids and membrane proteins to the plasma membrane via exocytosis, but the mechanisms and signaling pathways that regulate this coordination are unknown. Prior to neurite initiation in cortical neurons, the conserved Ena/VASp family of actin regulators mediates filopodia formation, and in the absence of Ena/VASp function, neurons fail to form both filopodia and neurites in culture as well as in the cortex. Adhesion to Laminin rescues both filopodia and neurite formation. Here we investigate the signaling pathway and coordination of cytoskeletal dynamics and exocytosis during Ena/VASp-dependent and Laminin-dependent neuritogenesis in embryonic cortical neurons. Using pharmacological agents and dominant negative approaches, we show that Laminin-dependent neuritogenesis requires the activity of Integrin receptors as well as the signaling molecules focal adhesion kinase, Src, and Rac. We find the activation of this signaling pathway results in a switch in the actin regulatory proteins necessary for neuritogenesis, from Ena/VASp to the Arp2/3 complex, as well as a switch in the v-SNARE mediating exocytosis, from VAMP2 during Ena/VASp-dependent neuritogenesis to VAMP7 during Laminin-dependent neuritogenesis. Using live cell total internal reflection fluorescence microscopy, we find that vesicle dynamics and fusion with the basal plasma membrane are modulated by adhesion to Laminin, and fusion mediated by VAMP2 and VAMP7 require the activity of Ena/VASp and the Arp2/3 complex, respectively. This novel coordination of the cytoskeleton and exocytosis is integral to neuritogenesis, and is likely to have implications in later stages of nervous system development as well.

1025/B183

**The WAVE/SCAR Complex Regulates α-Catenin Accumulation at Apical Membranes during Embryonic Morphogenesis.**

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α-catenin was thought to be a bridge linking adherens junctions to F-actin at the apical regions of epithelial cells. However, molecules of α-catenin cannot bind simultaneously to both F-actin and to its adherens junction partner, β-catenin, suggesting this is a dynamic and complex bridge. It has been proposed is that there is an antagonistic interaction between α-catenin and Arp2/3 during the establishment of cell-cell contacts, as both compete to bind F-actin. This suggests that altering the amount of available α-catenin will affect the formation of adherens junctions, and the connection between these junctions and apical F-actin. In this study we investigate how Arp2/3-dependent accumulation of F-actin at apical regions of epithelia contributes to the abundance and localization of adherens junction molecules at the plasma membrane. In our model system, the developing *C. elegans* intestinal epithelium, Arp2/3-dependent actin nucleation is required for apical F-actin enrichment and to regulate the size of the apical domain. While apical polarity appears to be established normally, over time apical defects progressively worsen, suggesting a defect in maintenance of cell organization. We therefore examined the abundance, apical accumulation and overall levels of apical junction proteins in the *C. elegans* intestinal epithelia in embryos and adults. We find that removing Arp2/3-dependent actin nucleation in these cells results in an overall drop in the levels of α-catenin, and a drop of α-catenin accumulation at the apical junctions in embryos and in adults. Subcellular fractionation reveals that loss Arp2/3-dependent actin nucleation shifts α-catenin from membrane-bound to cytoplasmic pools. In addition, the accumulation of other adherens junction molecules is altered. This study shows that α-catenin abundance and localization is being regulated by Arp2/3-dependent actin nucleation. One attractive interpretation is that Arp2/3-dependent actin
nucleation helps maintain epithelial polarity both during embryonic development and in mature epithelia by ensuring there is a sufficient pool of apical, membrane-localized α-catenin.

1026/B184
Filamentous Actin Flow and Turnover at Cell-Cell Contacts and Non-Contacting Membranes Are Different.
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Cells depend on the plasticity of their actin cytoskeleton meshwork to migrate and interact with other cells. Detailed studies of filamentous actin (F-actin) dynamics in migrating cells have identified mechanisms involving F-actin polymerization/depolymerization and flow at the leading edge of extending plasma membranes. However, little is known about actin organization and dynamics at contacting plasma membranes during the establishment of cell-cell adhesions. Previous studies suggested a direct link between the actin cytoskeleton, a complex of adaptor proteins (α- and β-catenin) and cadherin cell-cell adhesion proteins, but biochemical studies indicated a more dynamic interaction in which the actin cytoskeleton is not directly linked to the cadherin/catenin complex, but its architecture and dynamics would be regulated by the complex. We sought to compare F-actin dynamics at a free, non-contacting surface and at a nascent cell-cell contact in Madin-Darby canine kidney (MDCK) cells transiently transfected with mApple-actin using Total Internal Reflection-Fluorescent Speckle Microscopy (TIR-FSM). We tracked single-speckle trajectories of actin and observed that the retrograde F-actin flow was faster at cell-cell contacting membranes than at the free, non-contacting surface. We also observed higher actin turnover rate at the free, non-contacting surface compared with cell-cell contacts. Actomyosin contraction may be responsible for faster F-actin flow at the cell-cell interacting region than at the free, non-contacting surface. Inhibition of Arp2/3-dependent F-actin branching by α-E-catenin or of F-actin depolymerisation by Eplin may impede actin turnover at cell-cell contacts. These preliminary results provide evidence of different F-actin dynamics at cell-cell contacts and at the free, non-contacting membrane, and suggest that the underlying mechanisms may be adapted to the specific requirements of cell-cell adhesion and cell migration.

1027/B185
Regulated Src Kinase Activity Directs Invadopodia Maturation.
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Invadopodia are ventral membrane protrusions produced by metastatic cancer cells that degrade extracellular matrix (ECM). The steps in progression from pre-invadopodia (non-degradative stage) to functional, mature invadopodia are undefined. Cortactin is an actin-binding protein phosphorylated by Src kinase, and is a core invadopodia component. Knockdown of cortactin expression results in decreased invadopodia formation and MMP secretion, however the precise role of cortactin in maturation is unclear. To evaluate the role of cortactin in invadopodia downstream of Src, a tagged temperature sensitive v-Src mutant (tsLa29-GFP) was expressed in Src-deficient (SYF) fibroblasts. Invadopodia-like structures form at the permissive temperature and contain tyrosine-phosphorylated cortactin, but fail to degrade ECM indicating they remain in a pre-invadopodia state. To further evaluate this finding, SYF cells, and cSrc-restored SYF cells (SYF+/+) were transfected with tsLa29 or activated cSrc (cSrc527F). Expression of either active Src construct leads to pre-invadopodia formation in SYF cells, but induces mature invadopodia in SYF+/+ cells. To verify the apparent requirement for WT Src in invadopodia maturation, cSrc527F and WT cSrc were co-expressed in SYF cells. Cells containing both Src forms produce mature invadopodia that recruit endogenous membrane type-1 matrix metalloproteinase (MT1-MMP) to degradation sites. To evaluate the impact of Src-mediated cortactin phosphorylation on
invadopodia maturation, SYF+/+ cells stably expressing wild-type (WT) and phosphorylation-null (TYM) cortactin constructs were produced and transformed with cSrc527F. Cells expressing cortactin WT and TYM localize cortactin to mature and pre-invadopodia, respectively. These findings indicate that while elevated Src kinase activity per se is required to target tyrosine-phosphorylated cortactin to pre-invadopodia, regulated (i.e., cyclical) Src activity is necessary for cortactin to promote MT1-MMP targeting and matrix degradation activity. Use of the SYF/SYF+/+ system allows for the identification of key factors responsible for regulation of the Src/cortactin complex as pre-invadopodia complexes transition into mature invadopodia.

1028/B186
Anchoring of Vinculin to the Lipid Membrane Influences Cellular Mechanics.
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Vinculin is an important focal adhesion protein that modulates cell adhesion and migration. Its interaction with lipid membranes is believed to be necessary to ensure these processes [1]. We investigated two previously identified lipid binding sites located on vinculin’s tail: helix 3 (H3) and the C-terminus (CT) in In Vitro and In Vivo experiments. Using differential scanning calorimetry (DSC), we determined the lipid binding ability of the following synthesized protein constructs: H3wildtype (residues 944-972), H3mutant (K952, K956, R963, R966 to Q), CTwildtype (residues 1046-1066), CTmutant (R1060, K1061 to Q) and CT(pY1065). Results from DSC measurements show equal association of H3wildtype and H3mutated protein constructs with lipid vesicles consisting of DMPC/DMPS (70:30) and lipid vesicle insertion of CT in the following order: CTwildtype > CTmutant ~ CT(pY1065). To determine their mechanical influence, we transfected mouse embryonic fibroblasts deficient of vinculin (MEFvin-/-) expressing these vinculin-tail constructs linked to EGFP. Using 2D traction microscopy, we found that MEF(H3wildtype) and MEF(H3mutant) cells generate similar forces and that MEFvin-/- cells expressing CT(pY1065) generate higher forces compared to CTmutant and CT(Y1065 to F) cells. These observations show that the association of the H3 region of vinculin is not involved in cellular mechanical behavior and that, both the lipid insertion of the C-terminus of vinculin and its phosphorylation at Y1065 are important for cellular mechanical regulation [2]. [1] Diez et al. BBRC 373: 69-73 (2008) [2] Möhl et al. Cell Mot. Cytoskeleton 66: 350-364 (2009) This work was funded by DAAD, BaCaTeC and BFHZ.

1029/B187
Cluster Size Regulates Protein Sorting in the Immunological Synapse.
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During an immune response, receptors on the T cell engage ligands on the antigen-presenting cell and organize into a series of concentric rings collectively known as the immunological synapse (IS). In this pattern, large clusters of T cell receptors (TCR) congregate at the center of the interface, surrounded by a ring of the adhesion molecule leukocyte function-associated antigen-1 (LFA-1). This complex protein organization is known to regulate cell activation and signaling. Although actin polymerization is known to mediate receptor transport, the mechanism behind spatial sorting of proteins remains unclear. Here we investigate the role of protein cluster size in protein sorting using the hybrid live T cell-supported membrane system. We alter the clustering state of LFA-1, either by direct antibody crosslinking or by crosslinking its ligand, ICAM-1, on the supported bilayer. Using fluorescence microscopy, we observe the higher degrees of LFA-1 clustering lead to progressively more central localization of LFA-1 with the most clustered species colocalizing with TCR. Topographic imaging with reflection interference contrast microscopy (RICM) indicates that the large difference in length between the bound ectodomains of LFA-1 and TCR is maintained at the central zone. These results demonstrate that cluster size
is the predominant parameter in determining protein spatial positioning in the IS. We propose a sorting mechanism, based on frictional coupling to the actin cytoskeleton, that is consistent with these observations and may be generalized to all the cell surface proteins in the IS.

1030/B188
Spatial Organization of Ezrin at the Immunological Synapse.
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T cells of the immune system detect pathogen-deriv ed peptides on the surface of infected cells. The immunological synapse (IS) is the specialized signaling junction that forms between the two cell types. T cell receptors (TCRs) trigger and form clusters, which are transported to the center of the IS by the actin cytoskeleton within five minutes of contact. Once at the center, TCR clusters are endocytosed and down-regulated. However, at low concentration of ligand, TCRs trigger and cluster, but are not transported to the center. Here we investigate the mechanism of control of TCR cluster transport. Using a hybrid live cell-supported lipid bilayer system and immunofluorescence microscopy, we compared TCR colocalization with downstream signaling proteins. We identified that Ezrin (pY146) co-localization with TCR decreases with lower concentration of TCR ligand. Ezrin (pY146) is a membrane-cytoskeleton linker in several mammalian cell types, and directly interacts with actin and the TCR downstream kinase ZAP-70. We observed that Ezrin (pY146) co-localization with TCR correlates with the amount of TCR transport to the center of the IS. This observation suggests that Ezrin (pY146) controls TCR transport to the center and thus enhances TCR down-regulation. Interestingly, we also found that Ezrin (pY146) displays a heterogeneous radial distribution, with larger clusters in the periphery of the IS and smaller clusters in the center. This heterogeneous organization of Ezrin reveals the intricate spatial regulation of signaling at the immune synapse.

1031/B189
Ezrin Interactions with Biomimetic Vesicles Containing Phosphatidylinositol(4,5) Bisphosphate.
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The plasma membrane-cytoskeleton interface is a dynamic structure, participating in a variety of cellular events including cell shape, polarization and motility. Among the proteins involved in the direct linkage between components of the cytoskeleton and the plasma membrane is the ezrin/radixin/moesin (ERM) family. The FERM domain in their N-terminus contains a phosphatidylinositol 4,5 bisphosphate (PIP2) binding site responsible for membrane-binding whereas their C-terminus bind actin. In this work, our aim was to quantify the interaction of ezrin with large unilamellar vesicles (LUVs) containing PIP2 and with giant unilamellar vesicles (GUVs) containing PIP2. We first synthesized human recombinant ezrin bearing a cysteine residue at its C-terminus for subsequent grafting with Alexa488 maleimide. This allowed us to perform fluorescence spectroscopy and microscopy experiments. The functionality of labeled ezrin was checked by comparison with that of wild type (WT) ezrin. The affinity constant between ezrin and LUVs, determined by co-sedimentation assays and fluorescence correlation spectroscopy, was found to be ~5 µM for PIP2-LUVs and 20 to 70 lower for PS-LUVs, depending of the experimental technique. We found that the interaction is not cooperative for PIP2-LUVs. We prepared fluorescently labelled GUVs using PIP2 analogues used as tracers (0.1% of total lipids) to investigate ezrin/GUVs interactions by fluorescence microscopy. Zeta potential measurements confirmed that the effective incorporation of PIP2 in the GUVs. Finally, the interaction of ezrin with PIP2-containing GUVs was investigated. Using either labeled ezrin and unlabeled GUVs or both labeled ezrin and GUVs, we evidenced that clusters containing both PIP2 and proteins are formed.
1032/B190
Phospho-Regulation of ACAP4-Ezrin Interaction by Camp-Dependent Kinase Orchestrates Histamine-Stimulated Parietal Cell Secretion.

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The ezrin-radixin-moesin proteins provide a regulated linkage between membrane proteins and the cortical cytoskeleton, and also participate in signal transduction pathways. Ezrin is localized to the apical membrane of parietal cells and couples the PKA activation cascade to the regulated HCl secretion. Our recent proteomic study revealed a protein complex of ezrin-ACAP4-ARF6 essential for volatile membrane remodeling (Mol. Cell Proteomics. 2006. 5, 1437-1448). However, it has remained elusive as whether ACAP4 physically interacts with ezrin and how their interaction is integrated into membrane-cytoskeletal remodeling. Here we provide the first evidence that ezrin interacts with ACAP4 in PKA-mediated phosphorylation-dependent manner. ACAP4 locates in the cytoplasmic membrane in resting parietal cells but translocates to the apical plasma membrane upon histamine stimulation. ACAP4 was precipitated with ezrin from secreting but not resting parietal cell lysates, suggesting a phospho-regulated interaction. Indeed, this interaction is abolished by phosphatase treatment and validated by an In Vitro reconstitution assay using phospho-mimicking ezrinS66D. This phospho-mediated interaction was mapped to the N-terminal 400 amino acids of ACAP4. Importantly, ezrin specifies the apical distribution of ACAP4 in secreting gastric parietal cells as either suppression of ezrin or overexpression of non-phosphorylatable ezrin prevents the apical localization of ACAP4. In addition, over-expressing GAP-deficient ACAP4 results in an inhibition of apical membrane-cytoskeletal remodeling and parietal cell acid secretion. Taken together, these results define a novel molecular mechanism linking ACAP4-ezrin interaction to polarized epithelial secretion.

1033/B191
Shallow Curvature Realigns Actin and T Cell Receptor Transport.

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The actin cytoskeleton at intercellular junctions controls contact area shape, membrane protein organization and transport, and membrane signaling complexes. The immunological synapse between T cells and pathogen-presenting cells exemplifies the role of actin in reorganizing signaling molecules at the cell-cell interface and its control over immune response. However, the physical properties of actin and its interactions with membrane receptors at cell-cell junctions have not been characterized. We find that regions of shallow curvature (five microns radius of curvature) realign actin and surface receptors transport, and lead to elongation of contact zone across curvature topography. Primary T cells conform to regions of curvature at hybrid T cell-fluid supported lipid membrane junctions. The radial symmetry of the immunological synapse contact zone, micron-scale protein patterns and actin breaks, and elongates across regions of curvature, proportionally to their frequency. Single particle tracking of T cell receptors (TCRs) and gfp-actin revealed decreased transport across regions of curvature, which breaks the immunological synapse symmetry. Surprisingly, TCR and actin preferentially localize to and move along the precise location of curvature, suggesting a direct remodeling of actin by shallow curvature. This g- and f-actin enrichment to regions of curvature is not dependent on the presence of a specific intercellular protein binding pair, supporting the conclusion of a direct link between surface curvature and actin remodeling. Overall, these results emphasize the role of shallow curvature in altering actin, membrane protein and contact zone organization at intercellular junctions.
Macrophages use macropinocytosis to internalize small molecules and proteins. These internalized solutes are then used for metabolism or antigen presentation. Recently, the F-BAR family of membrane deforming and cytoskeleton-recruiting proteins has been established. One subfamily includes Cdc42 interacting protein-4 (CIP4), Toca-1, and FBP17, which are involved in endocytosis. Our laboratory discovered CIP4 in a yeast two hybrid screen with the Src kinase Lyn as bait. The objective of our study is to identify the unique properties of CIP4 in endocytic processes. Because CIP4 is the only subfamily member containing a Src phosphorylation site (Tyr471), we hypothesized that Src kinases might regulate macropinocytosis through phosphorylation-dependent events. We observed that CIP4 knockdown, but not that of Toca-1 or FBP17, resulted in decreased macropinocytosis of FITC-dextran in RAW264.7 cells, a macrophage cell line. In transient transfection of 293T cells, we demonstrated that v-Src, but not v-Abl, phosphorylates CIP4. Mutational analysis discerned CIP4 is phosphorylated on tyrosine 471 by v-src. Treatment of RAW264.7 cells with dasatinib (a Src/Abl inhibitor), but not imatinib (an Abl inhibitor) resulted in decreased macropinocytosis. Overexpression of v-Src increased macropinocytosis. Co-expression of v-Src increased the binding of CIP4 with either WASp or Dynamin-2. Our data supports a model whereby CIP4 is distinct from other F-BAR proteins in containing a Src phosphorylation site. Src-mediated phosphorylation of CIP4 and its binding partners WASp and Dynamin-2 facilitate macropinocytosis. This model predicts that in addition to the F-BAR domains, protein phosphorylation contributes to membrane deformation.

N-Terminal Basic Domain Participates in Plasma Membrane Targeting of Formin mDia2.

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Formins are auto-inhibited proteins that upon activation can nucleate and elongate actin filaments by processively binding their barbed ends. Formin mDia2 facilitates the formation of lamellipodia and filopodia that are necessary for locomotion. Active mDia2 localizes to the interface between the actin filament barbed ends and the plasma membrane (PM) and interaction with both surfaces is thought to control its subcellular localization. mDia2 binds barbed ends via its FH2 domain, but how it binds the PM is not clear. One idea is that the GTPase-binding domain (GBD) of mDia2 binds active small GTPase(s) at the PM. However, other mDia2 domains are also needed for its PM localization. To better understand actin-independent mechanisms of mDia2 targeting to the PM, we undertook a domain-based approach focusing on mDia2 domains other than FH2. We show that the PM targeting specificity resides in the amino terminus of mDia2, which contains four characterized subdomains: GBD, Diaphanous inhibitory domain (DiD), dimerization domain (DD), and coiled-coil (CC). In addition, we noticed a highly positively charged region (pI>10) at the very N-terminus of mDia2, which we called the basic domain (BD) and investigated its role in mDia2 targeting to the PM. By confocal microscopy and biochemical fractionations, we show that BD is targeted to the PM to a significant extent, although not as potently as the entire N-terminus (BD-GBD-DD-CC). Furthermore, deletion of BD from the mDia2 N-terminus (GBD-DD-DD-CC) severely compromises its PM localization, but does not completely abolish it, suggesting the existence of another PM-binding site within the N terminus. Since basic domains of other proteins can target them to the PM by direct interaction with lipids, we tested whether BD of mDia2 also has this capability. We show that BD binds acidic, but not neutral or basic, phospholipids in a dot-blot assay and is pelleted with liposomes containing negatively charged lipids. Together, our data suggests that BD of mDia2 significantly contributes to membrane localization of mDia2 through electrostatic association with acidic phospholipids in the PM. Supported by NIH grants GM70898 and HD07516.
1036/B194
FLJ22582 - A Novel I-Bar Domain Protein Expressed in Specialised Epithelial Cells.
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The actin cytoskeleton plays a central role in a number of cellular processes involving membrane dynamics. However, the exact mechanisms by which the polymerizing actin filaments interact with cellular membranes remain poorly understood. A central group of proteins functioning at the interface between plasma membrane and the actin cytoskeleton are the I-BAR family proteins. Mammals have five I-BAR family proteins, which all contain an N-terminal membrane binding I-BAR domain and an actin monomer binding WH2 domain at the C-terminus. All I-BAR domains studied so far - MIM, IRSp53, IRTKS and ABBA - induce filopodia formation when expressed in cells and deform PI(4,5)P2-rich membranes to tubular structures in vitro. We show that, in contrast to four other relatively widely expressed I-BAR proteins, the fifth member of the family, FLJ22582, is only expressed in the epithelial cells of the bowel and in the kidney. Interestingly, the biochemical properties of the FLJ22582 I-BAR domain differ from the other I-BAR domains. Electron tomography analysis revealed that FLJ22582 I-BAR domain induces a formation of narrow sheet-like structures and does not deform membranes into tubular structures like other I-BAR domains. We also determined the crystal structure of the I-BAR domain of FLJ22582. The structure displays significant differences, both in charge distribution and overall bending, compared to the I-BAR domains of MIM and IRSp53. The biochemical data and crystal packing contacts suggest a mechanism of cooperative self-association during membrane binding. Thus, FLJ22582 appears to regulate membrane dynamics through at least partially different mechanism compared to other I-BAR domain proteins.

1037/B195
Distinct Roles of PAR Proteins in Regulating Dynamic Myosin Network Assembly in Drosophila melanogaster Apical Constriction.
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Apical constriction is a major mechanism underlying tissue internalization during development. This constriction typically requires actomyosin contractility. Defining the mechanics and regulation of actomyosin assemblies is thus an important part of understanding the molecular bases of development. We have analyzed connections between myosin and the polarity regulators PAR-6, aPKC, and Bazooka (PAR-3) (the PAR complex) during amnioserosa apical constriction as dorsal closure occurs during Drosophila embryogenesis. Through immunofluorescence and live-imaging, we find that both the PAR complex and myosin accumulate at the apical surface of amnioserosa cells at dorsal closure—the PAR complex forming a patch of puncta and myosin forming an associated network. Mutations in baz, par-6, and apkc worsen the terminal cuticle phenotype of myosin mutants. These genetic interactions indicate that the PAR complex supports myosin activity during dorsal closure, and during other tissue morphogenesis in the embryo. We find that actomyosin contractility in amnioserosa cells is based on repeated assembly and disassembly of dynamic apical actomyosin networks, with each assembly event driving constriction of the apical domain. During dorsal closure, these networks cycle with a defined duration (170±84s) and a regular lull time (83±78s) between pulses. As the networks assemble they translocate across the apical patch of PAR complexes, which continually persist at the apical domain. Through loss-of-function and gain-of-function studies, we find that different components of the PAR complex regulate distinct phases of the actomyosin assembly-disassembly cycle, with Baz promoting the duration of actomyosin pulses and PAR-6/aPKC promoting the lull time
between pulses. These results identify the mechanics of actomyosin contractility with dynamic networks that drive amnioserosa apical constriction and how specific steps of the contractile mechanism are regulated by the PAR complex.

1038/B196
**Influence of Water Fluxes on Cell Morphology and Motility.**

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Water fluxes over the plasma membrane are mainly driven through water channels known as aquaporins (AQPs). Today thirteen mammalian AQPs are known and they influence a number of processes including cell motility. AQP9 is present in human leukocytes, which are highly motile cells, and when it is expressed in fibroblasts these develop a highly filopodial phenotype. Using stably transfected AQP9-GFP cell lines and various imaging techniques we show here that AQP9 induces highly dynamic filopodia in different cell types. It also augments their motility, adhesiveness, polarization and sensitivity to hyper-osmotic changes, which is accompanied by an increase in β2-integrin expression and a co-localization with the scaffolding protein α-dystrobrevin. We suggest that the increased water fluxes that occur at the site of AQP insertion enhance the cell ability for shape changes and thereby enhance its migratory capacity. Furthermore, the increased surface area that follows induction of filopodia enlarges the area for insertion of adhesion molecules and thus the attachment. Moreover, this process is promoted by co-localization with α-dystrobrevin, which could help anchor AQP9 to membrane micro-domains through a PDZ-motif interaction allowing polarized distribution of the water channels. We also present a hypothetical working model for AQP9 induced filopodia based on the interplay between increased water flux and other proteins interacting with AQP9 to enable polarized distribution of this protein together with other players involved in cell adhesion and motility.

1039/B197
**Membrane Tubulation and Actin Polymerization Induced by the EC/F-BAR Domain Protein PACSIN2 for Caveolae Formation.**

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The extended Fes-CIP4 homology (EFC) / FCH-BAR (F-BAR) domain protein, FBP17 or Toca-1, deforms membranes and activates N-WASP to induce actin polymerization for rapid clathrin-mediated endocytosis. The activation of the native form of N-WASP, the N-WASP-WIP complex, was achieved by the association with FBP17/Toca-1 and the membrane of a relatively large liposomes. on the other hand, caveolae are invaginations of the plasma membrane that contact actin filaments and have a longer lifetime than clathrin-coated pits. Cavin proteins and caveolin are involved in caveolae biogenesis. Here we examined the function of the EFC/F-BAR protein pacsin2. Pacsin2 generates membrane invaginations for caveolae before the recruitment of PTRF/Cavin-1. The invagination by pacsin2 was enhanced by the loss of N-WASP-mediated actin filament induction, indicating the role of actin filaments in the appropriate depth of caveolae. Moreover, the N-WASP-induced actin polymerization by pacsin2 in the presence of membranes was 2.5-fold higher than the basal activity, and less than one-tenth of that by Toca-1. FRAP analyses revealed the faster turnover of pacsin2 than FBP17 on invaginations. These characteristics of pacsin2 suggested that the actin filaments induced by pacsin2 are weakly coupled to caveolae in a manner insufficient for vesicular fission, but are sufficient for appropriate caveolae formation.

1040/B198
**Role of F-BAR Proteins Cdc15p and Bzz1p in Fission Yeast Endocytosis.**
Endocytosis by yeast cells depends on highly coordinated, local assembly and disassembly of a dense network of actin filaments associated with clathrin-coated vesicles. Genetic analysis and live cell microscopy have identified at least 50 proteins that are necessary for endocytosis including clathrin, clathrin-interacting proteins, actin and actin binding proteins. These proteins assemble in a highly ordered and regulated fashion starting with the recruitment of clathrin and its associated proteins, followed by the recruitment of proteins required for actin assembly (Wsp1p, Myo1p, Arp2/3 complex) and actin turnover (Act1p, Acp2p, Crn1p). Here we show that *S. pombe* F-BAR proteins, Cdc15p and Bzz1p make important contributions to this process by differentially interacting with and regulating the activities of Myo1p and Wsp1p. Previously, it was shown that Wsp1p and Myo1p define two independent pathways of actin assembly in vivo. Both our microscopy and genetic interaction data place Bzz1p in the Wsp1p pathway and Cdc15p in the Myo1p pathway. At their peak local concentrations, the stoichiometries of the proteins in actin patches were 2:1 Wsp1p:Bzz1p and 1:1 Myo1p:Cdc15p. We used fluorescent proteins fused to actin patch protein coronin1 (Crn1p) to determine how the absence of Bzz1p or depletion of Cdc15p influences endocytosis. We observed two defects in both mutants. First, Crn1p-mEGFP patches were less polarized than in wild type cells. Second, Crn1p-mEGFP patches moved away from the plasma membrane much slower in absence of Bzz1p or the depletion of Cdc15p similar to their behavior in strains lacking Wsp1p or Myo1p. On the other hand, neither the absence of Bzz1p nor the depletion of Cdc15p (41xnmt1 repressible promoter) changed the amount of Wsp1p, Myo1p or Arp5 subunit of Arp2/3 complex recruited to actin patches. In spite of normal levels of Wsp1p, Myo1p and Arp2/3 complex in actin patches, bzz1Δ and 41xnmt1cdc15 cells polymerized 3- to 5-fold less actin in patches than wild type cells. Based on these results we hypothesize that F-BAR proteins promote assembly of actin filaments via the activation of Wsp1p and Myo1p pathways and may also facilitate the vesicle scission event at the end of endocytosis.

Filopodia are rod-shaped cell surface protrusions comprised of parallel actin filament bundles and have been implicated in many important cellular functions such as sensing of guidance cues during cell migration, cell adhesion or phagocytosis. These highly dynamic structures grow by deforming and pushing the plasma membrane outwards while actin monomers are being incorporated at their tips. However, as yet it has not been conclusively clarified whether the sole force of actin polymerization is sufficient to drive membrane protrusion or whether other factors such as membrane protrusion inducing proteins are required for this process. In mammalian cells, these belong to a multi-gene family of inverse BAR-domain (I-BAR) containing proteins also referred to as IMD (IRSp53/MIM-domain)-proteins. To address this issue in a less complex system, we analyzed the physiological relevance of the single I-BAR/IMD-containing protein DdMIM in Dictyostelium. Indirect immunofluorescence revealed that DdMIM accumulates prominently to large vacuoles that are frequently connected by tubular structures as well as to punctuate structures beneath or at the plasma membrane corroborating a regulatory role of DdMIM in lipid dynamics. To evaluate a potential contribution of DdMIM to filopodia formation, we disrupted the DdMIM encoding gene by homologous recombination. The null-mutant showed defects in various cellular processes such as growth and establishment of cell morphology, however, filopodia formation was not impaired as assessed by F-actin labelling of fixed specimens and live cell imaging. Together, our results suggest that DdMIM is not required for filopodia formation.
demonstrate that at least in the Dictyostelium system, a membrane-deforming activity of I-BAR domain-containing proteins is dispensable for filopodia formation.

1042/B200
Kinesin Is an Essential Anterograde Microtubule Motor Protein for FcγR-Mediated Phagocytosis in RAW264.7 Mouse Macrophages.

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Fcγ Receptor (FcγR)-mediated phagocytosis is a cellular event that is evolutionary conserved to digest IgG-opsonized pathogens and senescent red blood cells. The retrograde microtubule motor protein, dynein has been shown to be important in trafficking the nascent phagosome towards the central lysosomes for degradation by hydrolytic enzymes. Based on our results, we postulate that kinesin, an anterograde microtubule motor protein is involved in forming the phagosome, by trafficking endomembrane vesicles and phagocytic receptors to the localized phagocytic cup. Our experiments consisted of transfecting RAW 264.7 murine macrophages with a dominant negative kinesin construct (EGFP-Kif5B-DN) and assaying particle binding and phagocytosis. We first observed a significant decrease in particle binding and internalization of IgG-coated sheep RBCs (IgG-sRBC) in cells transfected with Kif5B-DN. There was no difference in lysosomal-associated membrane protein (LAMP-1) distribution on phagosomes in EGFP-Kif5B-DN-transfected cells compared with control cells revealing proper maturation during phagocytosis. SEM analysis showed abnormal pseudopod formation that correlated with the decrease in IgG-sRBC binding. To investigate trafficking to phagocytic cups, delivery of endomembrane markers, such as Rab11 and VAMP7 was studied. While VAMP7 dynamics were normal, Rab11 trafficking to the phagocytic cup was disrupted in EGFP-Kif5B-DN-transfected cells. Recycling of membranes from nascent phagosomes was also examined and we observed a marked disruption of trafficking of membranes resulting in a net loss in Fcγ receptors on the cell surface. In conclusion, anterograde trafficking via the conventional kinesin motor is essential for receptor and membrane delivery necessary for IgG-particle binding and pseudopod formation.

1043/B201
Adenomatous Polyposis Coli Regulates Huvec Adhesion and Migration Independently of Its Roles in β-Catenin Signaling and Transcriptional Activity.

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Adenomatous polyposis coli (APC) is a tumor suppressor commonly mutated in cancers. APC has two major functions: 1) an organizer of actin and microtubule cytoskeletons, and 2) a regulator of the Wnt signaling effector β-catenin. It is unclear whether these two functions are convergent or divergent. We are using primary Human Umbilical Vein Endothelial Cells (HUVEC) to investigate the interplay between these two APC functions. Endogenous APC localizes to two distinct sub-cellular sites: 1) in punctate clusters localized at the tips of membrane extensions and occasionally at cell-cell contacts, which requires microtubules, but not actin, and 2) along the plasma membrane with the VE-cadherin/catenin complex at sites of cell-cell contacts, which requires actin but not microtubules. Significantly, APC can exchange between these two sub-cellular pools, as disruption of VE-cadherin-based adhesion abolishes APC cell-cell contact localization and results in a concomitant increase in the number and size of APC clusters. To identify APC functions in β-catenin degradation and nuclear signaling, we examined APC/β-catenin phosphorylation. Sequential phosphorylation by casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK3β) targets APC and β-catenin for degradation by the proteasome. Significantly, we find high basal levels of CK1- and GSK3β-phosphorylated APC and β-catenin, and phospho-β-catenin is in a complex with APC, but not VE-cadherin, at cell-cell contacts and APC clusters. Accordingly, basal levels of Tcf/Lef transcription are low, indicating a highly active destruction complex that limits the amount of free β-catenin available for transcription. While CK1/GSK3β inhibition decreases the level of phosphorylated forms of APC and β-catenin, surprisingly we find no overall increase in the total level of β-catenin or Tcf/Lef transcriptional activity. Significantly, inhibition of CK1/GSK3β decreases the amount of APC localized to cell-cell contacts.
contacts and in clusters, and dramatically decreases the rate of cell migration. These data indicate that in HUVECs the phosphorylated forms of APC and β-catenin regulate cell-cell adhesion and migration independently of β-catenin transcriptional activity and Wnt signaling.

1044/B202

Association of an Eyespot Photoreceptor with Acetylated Microtubules in Cytoskeletal Mutants of Chlamydomonas reinhardtii. T. M. Mittelmeier, J. S. Boyd, C. Dieckmann; Molecular and Cellular Biology, University of Arizona, Tucson, AZ

We are investigating the relationship between the cytoskeleton and the asymmetrically localized eyespot of the green alga, Chlamydomonas reinhardtii. In Chlamydomonas, each of two basal body/centrosomes (mother and daughter) at the anterior of the cell nucleates the assembly of a flagellum and of two bundles of acetylated microtubules, termed “rootlets.” The eyespot, which comprises pigment granule layers in the chloroplast underlying rhodopsin-family photoreceptors in the plasma membrane, is associated specifically with one of the rootlets derived from the daughter basal body\(^1\). This association led to the hypothesis that localization of the eyespot, like that of a variety of cellular components, is determined by cytoskeletal structures. This hypothesis predicts that in cells with mutations that disrupt rootlet organization, the eyespot will be associated with aberrant rootlets as opposed to maintaining a specific location relative to other cellular structures. We used indirect immunofluorescence to label the ChR1 photoreceptor and acetylated microtubules in the following mutants with cytoskeletal defects: bld2 (ε-tubulin)\(^3\), vfl2 (centrin)\(^4\), mlt1 (multiple eyespot phenotype)\(^5\), and asq2 (tubulin co-chaperone Tbccd1)\(^6,7\). ChR1 was associated with rootlets in all strains. In many asq2 cells, a second aggregation of ChR1 was found associated with rootlets derived from the mislocalized daughter basal body. These data are consistent with the hypothesis that acetylated microtubules direct ChR1 localization. To investigate the hypothesis that the location of the photoreceptors directs placement of the plastidic pigment granule layers, we are examining localization of a pigment granule-associated protein, EYE3, in cells with aberrant patterns of ChR1. \(^1\)Holmes and Dutcher 1989 J Cell Sci 94:273-85 \(^2\)Kreimer 2008 Curr Genet 1:19-43 (review) \(^3\)Dutcher et al. 2002 Mol Biol Cell 13:3859-69 \(^4\)Taillon et al. 1992 J Cell Biol 119:1613-24 \(^5\)Lamb et al. 1999 Genetics 153:721-9 \(^6\)Feldman et al. 2007 Plos Biol 5:e149 \(^7\)Feldman and Marshall 2009 Curr Biol 19:1238-43

1045/B203

Arf6 Contributes to PMA Induced Podosome-Like Ventral Ruffle Formation by Influencing Endosomal Membrane Trafficking.
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Several studies support a role for the small GTPase, Arf6, in the invasive activity of a number of metastatic cancer cell lines. Here we show that a combination of increased Arf6 activity achieved through exogenous expression of Arf6 wt, Arf6 Q67L, or EFA6 (an Arf6 GEF), and treatment with the phorbol ester, PMA, induces the formation of large podosome-like ruffle structures located on the ventral surface of HeLa cells. These structures contain F-actin, cortactin, and src family kinases (SFK) and are highly dynamic. They can be inhibited by treatment with PKC inhibitors, or SFK inhibitors, or by expression of Rac1 T17N. Beas2b, a bronchial airway epithelial cell line, have recently been shown to form podosomes when treated with PMA that resemble the structures seen in HeLa cells. Indeed we found that treatment with PKC inhibitors, and SFK inhibitors, as well as expression of a dominant negative form of Arf6 inhibits the formation of these structures in Beas2b cells suggesting they are similar to the structures induced in HeLa cells. Arf6 associates with and regulates trafficking through a clathrin independent endocytic (CIE) pathway. We found that endogenous SFK, and the actin regulatory proteins, IQGAP, and drebrin associate with CIE membranes in HeLa cells. Futhermore, expression of Arf6 Q67L induces an accumulation of CIE derived membranes in many cell types. We found that SFKs, IQGAP, and drebrin associate with Arf6 Q67L induced internal vacuoles in both HeLa and Beas2B cells, and that treatment of HeLa cells expressing Q67L with PMA, or treatment of Beas2B cells with PMA leads to the redistribution of these proteins and associated endocytic membranes to the large
podosome like ruffles. This suggests that Arf6 and associated endocytic membranes might contribute to PMA induced podosome/ventral ruffle formation through regulating the localization of SFK, and actin regulatory proteins like IQGAP and drebrin.

1046/B204

WHAMM and Rab1 Cooperate to Promote Membrane Tubulation.

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The Arp2/3 complex is an actin nucleator that plays critical roles in diverse cellular processes. Its activity is controlled by nucleation-promoting factors (NPFs) including WASP/N-WASP, WAVE1-3, and WHAMM (WASP Homologue associated with Actin, Membranes, and Microtubules). Unlike other NPFs, WHAMM localizes to the cis-Golgi apparatus and tubulo-vesicular membranes that move in both an actin- and microtubule-dependent manner. WHAMM influences the organization of the Golgi, facilitates anterograde membrane transport, and functions in membrane tubulation. Whereas the WASP- and WAVE-family NPFs are known to be regulated by the GTPases Cdc42 and Rac1, a role for small GTPases in WHAMM function has not been explored. We therefore tested whether known ER- or Golgi-associated GTPases, including Sar1, Arf1, Cdc42, and Rab1, are involved in WHAMM-mediated membrane tubulation in cells. Of these, only wild type or constitutively active Rab1 stimulates both the formation and elongation of membrane tubules. Conversely, expression of dominant negative Rab1 mutants or depletion of Rab1 protein levels by RNAi suppresses WHAMM-associated membrane tubule formation. Importantly, WHAMM and Rab1 colocalize along dynamic tubulo-vesicular membranes that require both F-actin and microtubules for their movement, and inactivation of Rab1 disrupts WHAMM localization to membranes. Rab1 co-precipitates with the N-terminal WHAMM membrane-localization domain (WMD) from cell extracts, indicating that the two proteins interact. Collectively, these results demonstrate that WHAMM and Rab1 cooperate to promote membrane tubulation, and highlight a previously-unrecognized ability of Rab1 to influence membrane dynamics by signaling to cytoskeletal proteins.

1047/B205

Cortical Actin Controls Endocytic Scission to Stabilize Ingressing Cytokinesis Furrows.

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Cellularization of the syncytial fly embryo is a dramatic cytokinesis event that expands the plasma membrane and underlying cortex at rates of 0.5-1 micron per minute, but counterintuitive to the need for rapid cell surface growth is the occurrence of endocytosis at the tips of early, ingressing cellularization furrows. Endocytosis has been similarly observed at cytokinetic furrows in cell types ranging from yeast to mammals, but its function there is unclear. Goals of our current work include 1) understanding how endocytosis influences cell surface growth; and 2) identifying mechanisms that prevent endocytosis from undoing this growth. We previously reported that the novel gene nullo controls endocytosis at cellularization furrows. nullo mutations impair cortical F-actin accumulation and scission of endocytic vesicles, such that distended membrane tubules remain tethered to the plasma membrane, depleting structural components from furrows and precipitating furrow regression. We now find that a second novel gene, serendipity-α, likewise limits protein and membrane turnover at cellularization furrow tips by facilitating efficient endocytic scission, and so insures stable furrow progression. Serendipity-α shares structural homology with F-actin binding proteins, including Vinculin and α-Catenin; and Serendipity-α is required for cortical F-actin accumulation at cellularization furrows. Interestingly, while the phenotypes of nullo and serendipity-α mutants are qualitatively indistinguishable, our genetic analysis shows that they work in distinct, parallel pathways. Thus, this work suggests that endocytosis must be stringently controlled to support cell surface growth, and reveals that new actin cortex, built by multiple genetic pathways, can serve as a critical controller of endocytosis during furrow ingression.
Laminin-Based Cell Adhesion Anchors Microtubule Plus Ends to the Epithelial Cell Basal Cortex through LL5α/β.

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In polarized epithelial cells, microtubules are aligned along the apicobasal axis with their plus-ends facing toward the basal side; however, the molecular mechanisms underlying this polarization of the microtubule network remain to be clarified. LL5β has been identified as a microtubule-anchoring factor that attaches EB1/CLASP-bound microtubule plus-ends to the cell cortex. Here, we show that LL5β and its homologue LL5α (LL5s) are required to maintain the density of growing microtubules at the basal side of polarized epithelial cells. We analyzed the three-dimensional distribution of microtubule growth by visualizing EB1-GFP, a marker for growing microtubule ends, in fully polarized epithelial sheet using MCF-10A human mammary epithelial cells. RNA interference (RNAi)-based depletion of LL5s reduced the growing population of microtubules specifically at the basal cortex. LL5s are distributed at cell-substratum adhesion sites that are distinct from focal adhesions and colocalize with laminin-5 and its receptors, integrins α3β1 and α6β4. Depletion of these integrins abolished the basal localization of LL5s, and conversely depletion of LL5s diminished the amount of integrin α3 at the basal cell cortex. Furthermore, activation of integrin α3 is sufficient to initiate the LL5 accumulation at the cell cortex. Our findings reveal that the epithelial cell adhesion to the laminin basement membrane anchors microtubule plus-ends at the basal cortex through LL5s.

Gene Expression Profiles in Mouse Embryo Fibroblasts Lacking Stathmin, a Microtubule Regulatory Protein, Reveal Changes in the Expression of Genes Contributing to Cell Motility.

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Stathmin (STMN1) protein functions to regulate assembly of the microtubule cytoskeleton by destabilizing microtubule polymers. Stathmin over-expression has been correlated with cancer stage progression, while stathmin depletion leads to death of some cancer cell lines in culture. In contrast, stathmin-null mice are viable with minor axonopathies and loss of innate fear response. Several stathmin binding partners, in addition to tubulin, have been shown to affect cell motility in culture. To expand our understanding of stathmin function in normal cells, we compared gene expression profiles, measured by microarray and qRT-PCR, of mouse embryo fibroblasts isolated from STMN1+/+ and STMN1−/− mice to determine the transcriptome level changes present in the genetic knock-out of stathmin. Microarray analysis of STMN1 loss at a fold change threshold of ≥2.0 revealed expression changes for 437 genes, of which 269 were up-regulated and 168 were down-regulated. Microarray data and qRT-PCR analysis of mRNA expression demonstrated changes in the message levels for STMN4, encoding RB3, a protein related to stathmin, and in alterations to many tubulin isotype mRNAs. KEGG Pathway analysis of the microarray data indicated changes to cell motility-related genes, and qRT-PCR plates specific for focal adhesion and ECM proteins generally confirmed the microarray data. Several microtubule assembly regulators and motors were also differentially regulated in STMN1−/− cells, but these changes should not compensate for loss of stathmin. Approximately 50% of genes up or down regulated at a fold change of ≥2.0 in STMN1−/− mouse embryo fibroblasts function broadly in cell adhesion and motility. These results support models indicating a role for stathmin in regulating cell locomotion, but also suggest that this functional activity may involve changes to the cohort of proteins.
expressed in the cell, rather than as a direct consequence of stathmin-dependent regulation of the microtubule cytoskeleton.

1050/B208  
**Mmb1p, a Molecular Adaptor between Microtubules and Mitochondria.**  
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Proper distribution of mitochondria is critical for their inheritance during the cell cycle. In fission yeast the distribution of mitochondria appears to largely rely on the microtubule cytoskeleton. It also has been shown by electron microscopy that mitochondria are somehow connected to microtubules. Whether or not the growth and shrinkage of microtubules drives the distribution of mitochondria is still currently controversial and the detailed molecular mechanism underlying regulation of mitochondrial distribution is unclear. Here, we have identified a mitochondrial protein mmb1p, which is able to physically bind to microtubules. Interestingly, mmb1p highly concentrates in a region where mitochondria and microtubules overlap and the microtubule affinity of mmb1p is independent of microtubule dynamics. Loss of mmb1p causes aberrant mitochondrial aggregation at cell tips and influences microtubule dynamics while overexpression of mmb1p strongly enhances the alignment of mitochondria with microtubules. Moreover, our live cell imaging data shows that mitochondrial distribution is a consequence of the combination of complex mitochondrial dynamics and the mitochondrial dynamics is strictly dependent upon the microtubule scaffold. Strikingly, depletion of mmb1p causes severe loss of mitochondria when cells propagate, leading to a growing defect. Therefore, our work has revealed that mmb1p is an important molecular adaptor between microtubules and mitochondria, and the connection ensures proper mitochondria distribution.

1051/B209  
**Role of INF2 in the Formation of Stable Microtubules.**  
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Microtubules (MTs) are essential for the polarization of many cell types. There are two well characterized pools of MTs, dynamic and stable MTs. Stable are characterized by a long half-life and undergo posttranslational modifications such as detyrosination (GluMTs), which allow them to be recognized by motors and other MT interacting proteins. In 3T3 fibroblasts, stable GluMTs formation is triggered by lysophosphatidic acid (LPA) in serum through a pathway involving activation of Rho and its effector mDia. In these cells LPA also controls centrosome orientation towards the leading edge in a process regulated by Cdc42, the kinase MRCK, Par proteins and the dynein/dynactin complex. In this study we tested the requirement of INF2, a new formin with high homology with mDia proteins, in both stable GluMT formation and centrosome orientation in migrating NIH 3T3 cells. Using a siRNA approach we found that stable Glu MTs were substantially inhibited by INF2 siRNA depletion to levels comparable to mDia1 siRNA depletion. However, no inhibition of centrosome orientation towards the wound edge was observed when either INF2 or mDia1 were silenced. Immunolocalization of endogenous INF2 confirmed a reticular distribution overlapping with the ER and the MT cytoskeleton (Chhabra et al., JCS), suggesting a role for INF2 in regulating the association of ER membranes to MTs. Microinjection of serum-starved cells with an INF2 fragment consisting of the FH1FH2 domains induced formation of stable Glu MTs whereas INF2 full-length did not. Thus, it appears that the ability of INF2 to induce stable Glu MTs is autoinhibited in the intact INF2 molecule. Using In Vitro MT binding assays, we found that the carboxyl-terminal half of INF2, including the FH2 domain, binds to MTs directly and stabilizes them from cold induced depolymerization, as observed previously for the FH2 domain of mDia (Bartolini et al., JCB, 2008). These results are consistent with previous findings showing that the formation of stable Glu MTs and the reorientation of the centrosome are independent processes in NIH3T3 cells. In addition, they point to an involvement...
of multiple formins in the regulation of the stability of the MT cytoskeleton downstream of Rho GTPases.

1052/B210
Regulation of Microtubule Dynamics by the Fission Yeast XMAP215 Alp14p and the CLASP Cls1p.
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The XMAP215 and CLASP family proteins are conserved microtubule (MT) regulatory proteins that bind to tubulin dimers via TOG domains. XMAP215 proteins are MT-plus end binding (+TIP) proteins that are thought to act as MT polymerases (Brouhard et al., 2008), while CLASP appear to be lattice-associated proteins that function in MT rescue (Bratman and Chang, 2007; Al-Bassam et al., unpublished). Although well studied in vitro, detailed characterization of their in vivo function is far from complete. We are studying a XMAP215 homologue alp14p (Garcia MA et al., 2001, 2002; Sato M et al., 2004) and a CLASP homologue cls1p in fission yeast Schizosaccharomyces pombe. In interphase cells, alp14p localizes to the growing MT plus ends, while dis1p (the second XMAP215 homologue) binds along the MT lattice. alp14 null cells are viable but exhibit very short, misorganized interphase MTs. Using time-lapse microscopy and kymograph analysis with GFP-tubulin expressing strains, we measured the dynamic parameters of these interphase MTs. alp14 null mutants exhibited a 1.5x decrease in MT growth and 2x decrease in shrinkage rates. There was also a large increase in MT catastrophe and MT pause frequencies. Other MT regulatory factors such as mal3p (EB1 homologue) and tip1p (CLIP170 homologue) were localized normally in the absence of alp14p. The localization of alp14p at MT plus ends was independent of mal3p, tip1p, and tea2p (kinesin). Ongoing studies include using mutational analyses to dissect and compare the domains in alp14p and cls1p (CLASP homologue) to provide a mechanistic understanding of how these related proteins differentially regulate the dynamics of MTs.

1053/B211
Characterization of a Primary Rat In Vitro Model of Tau Hyperphosphorylation by Single Cell-Based Image Analysis.
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Tau is a microtubule-associated protein widely expressed in the nervous system, where it regulates the assembly and stability of neuronal microtubules. Hyperphosphorylation of tau is the precursor to the formation of neurofibrillary tangles which are characteristic in the pathogenesis of Alzheimer's disease. By treating embryonic rat cortical neuronal cultures with okadaic acid, a phosphatase inhibitor, this hyperphosphorylated state can be simulated in vitro. Tau is phosphorylated by a host of kinases at multiple sites. By performing immunocytochemistry utilizing a panel of phospho-tau and total tau antibodies, effects on tau phosphorylated sites and their location within the neuron can be monitored by image analysis. The Arrayscan VTI HCS platform algorithms allow for conversion of these multiplexed fluorescent images into numeric data which captures changes in tau intensity and location. In addition, a tubulin marker provides information on cytoskeletal parameters such as cell size, shape, and neuronal process length. To characterize this model, experiments were performed to assess the effects of modulating kinases on 1) Tau phosphorylation at six different sites; 2) Translocation of phospho-specific tau within cellular compartments; and 3) Effects on the tubulin structure within the cytoskeleton. It was discovered that kinase inhibitors had an effect on many of the phosphorylated sites, blocking the okadaic acid-induced hyperphosphorylation at varying degrees, most notably in the cell body compartment. Also highly affected was a cytoskeletal parameter, process (axonal) length, which retracts upon addition of okadaic acid. Pretreatment with a kinase inhibitor results in this effect being markedly attenuated. This assay has the potential to illuminate kinase interactions with tau which may help us to understand and evaluate possible therapeutic intervention to delay or prevent the onset of AD.
1054/B212

The Microtubule Plus End Tracking Protein CLASP Is a Substrate of the Abelson Protein Tyrosine Kinase.

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The Abelson (Abl) non-receptor tyrosine kinase is a central regulator in cell proliferation, morphology and movement. Genetic studies in Drosophila have indicated that Abl cooperates with the microtubule plus-end tracking protein CLASP in axonal targeting, however, the mechanism remains unknown. Here we show in vertebrate that Abl associates with CLASP. This interaction appears to be mediated by direct binding between Abl and CLASP, as it can be observed with purified proteins. Moreover, serum factors promote the association of Abl with CLASP2 and induce the tyrosine phosphorylation of CLASP2 in cultured cells, similar to that achieved with transfection of activated Abl-pp, both of which is abolished with treatment of STI-571. Using purified protein assays, we find that CLASP2 is a substrate of the Abl kinase. These and other observations suggest that Abl directly regulates the functional properties of CLASP-family proteins, consistent with genetic data showing that CLASP is required for Abl function during axon guidance.

1055/B213

CLASP2 Is Localized to the Meiotic Spindle and Germline P Granules in C. elegans.

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CLASP2 is a +TIP protein that is associated with the kinetochore during cell division and is found at the plus ends of microtubules in the leading edges migrating cells and growth cones. CLASP2 was localized by confocal microscopy using CLASP2 antibodies (the kind gift of A. Desai and K. Oegema). CLASP2 was localized to many cell types, including the apical junctions of intestinal cells, several strongly expressing somatic cells, and the nerve ring. CLASP2 labeled the meiotic spindle in newly fertilized eggs. In the hermaphrodite gonad, CLASP2 antibody (Ab) stained the rachis and the syncytial membrane. Staining was observed at the membrane of germline vesicles. Interestingly, CLASP2 Ab stained germline P granules throughout the life cycle: in oocytes, in embryos, in larval stages, and in the adult gonad. Colocalization of GFP-labeled P granules in the SS747 strain (the kind gift of S. Strome) with CLASP2 Ab staining was verified by confocal microscopy. Further, the specificity of CLASP2 Ab staining of all these structures was verified by competition with excess recombinant CLASP2 protein. Because P granules require a cytochalasin-inhibitable microfilament system for proper localization, it will be interesting to see what role CLASP2 plays in P granules.

1056/B214

TPX2-Eg5 Interaction Regulates Kinetochore Fiber Formation in Mammalian Cells.

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In the mitotic spindle, each kinetochore is linked to the spindle pole by a bundle of microtubules, the kinetochore fiber. Capture of dynamic centrosomal microtubules and formation of microtubules at, or near, kinetochores both contribute to kinetochore fiber formation in mammalian cells. TPX2 is a Ran-regulated, microtubule-associated protein that is required for microtubule formation at kinetochores. Using photoactivation of PA-GFP-TPX2, we found that TPX2 is transported poleward in a dynein, Eg5 and microtubule flux dependent manner. In Xenopus, recent work shows that the C-terminal 35 amino acids of TPX2 interact with the kinesin Eg5. Pull-down experiments with human TPX2 and Eg5 confirm a weak interaction that requires the C-terminus of TPX2. To determine if the TPX2-Eg5 interaction contributes to TPX2 transport
and kinetochore fiber formation in mammalian cells, we generated human TPX2 lacking the C-terminal 35 amino acids (TPX2-710). Photoactivation of PA-GFP-TPX2-710 showed that this domain is required for efficient poleward transport of TPX2. Further, we show that expression of TPX2-710 in mammalian cells results in a disorganized spindle phenotype, characterized by excessive microtubule formation near chromosomes, long astral microtubules and defects in chromosome alignment. In contrast, overexpression of full-length TPX2 induced spindle shortening, but did not stimulate microtubule formation near chromosomes. To further test if the TPX2-Eg5 interaction is important for spindle formation and function, we microinjected mitotic cells with bacterially expressed C-terminal 35 amino acids of TPX2. In microinjected cells, extensive astral microtubules, and microtubule bundles, were observed in the cell periphery. In anaphase cells, interzonal microtubule formation was defective. These results support a model in which the TPX2-Eg5 interaction is important for poleward transport of TPX2, for limiting microtubule formation near chromosomes.

**1057/B215**
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Plant cells have unique microtubule network; cortical microtubules, preprophase band, non-centrosomal spindle and phragmoplast. Microtubule-associated proteins (MAPs) are essential for organization and function of these networks. To understand cell biology of microtubule networks, we try to clarify overall picture of MAPs by proteomics. MAPs were purified from a crude extract of Arabidopsis cultured cell mini-protoplasts by two cycles of microtubule polymerization - depolymerization. Mass spectroscopy identified 1608 proteins in the MAP fraction (score > 20), including 74 % known MAPs (86 MAPs identified / 116 known MAPs) such as MAP65 family, kinesin family, MAP70 family, WDL1 family, katanin, MOR1, AIR9, CLASP, RUNKEL, SPR2 family, γ-tubulin complex. MAP fraction also contains many RNPs (ribonucleoproteins), which are involved in translation and RNA splicing and export, might interact with interphase microtubules networks. Other RNPs, which are involved in nucleolus functions and DNA replication and repair, might participate in spindle organization. In addition, MAP fraction contains peroxisome and Golgi/ER itself, suggesting that these organelle interact with microtubule network in plant cells. Dynamin families were also detected in MAP fraction. Moreover, specific enzyme families were concentrated in MAP fraction, indicating that several metabolic pathways act on microtubule networks. To characterize novel proteins in MAP fraction, we expressed 17 GFP-fused uncharacterized proteins in Arabidopsis plants. Half of them localized to nucleus, suggesting that these proteins are RNPs. One GFP-fused protein clearly labeled cortical microtubules, indicating that this protein is a novel MAP. Two GFP-fused proteins labeled intracellular particles, which move along ER and stop on cortical microtubules. In addition, faint small particles, which labeled one GFP-fused protein, move along microtubules. These proteins are MAP candidates. In conclusion, the potential MAP list would be great asset for future plant microtubule studies.

**1058/B216**
The Drosophila Spectraplakin Short-Stop, an Actin-Microtubule Cross-Linking Factor.
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In many cell types, directed migration requires a high degree of spatial and temporal coordination between the actin and microtubule cytoskeletal systems. Proteins that physically crosslink actin and microtubules are thought to play a significant role in this coordination. We are studying Drosophila Short-stop (Shot) the sole member of the spectraplakin family in flies. Spectraplakins contain N-terminal calponin homology (CH) domains that binds actin, a central rod composed of plakin domains and spectrin-like repeats, and a C-terminal GAS2 domain that binds microtubules.
These large, multi-domain proteins are thus poised to coordinate actin-microtubule crosstalk. Shot and its mammalian homologs display characteristic microtubule plus-end tracking behavior and interact with EB1. While genetic studies of Shot have previously revealed roles in cell-cell and cell-matrix adhesion, a detailed investigation into Shot’s role in coordinating actin-microtubule interactions has yet to be fully explored. We have conducted a detailed analysis of Shot behavior in Drosophila S2 cells and performed a structure/function study to determine the contribution of each domain to the dynamics of the protein. We used RNAi to examine cytoskeletal dynamics in Shot-depleted cells and found depletion of Shot uncouples the microtubule and actin cytoskeletons leaving microtubules impervious to the “piling-up” of microtubules caused by retrograde flow of actin within the lamellipodia. We have found that depletion of Shot increases the amount of sliding and “fishtailing” observed in acentrosomal microtubule array of S2 cells indicating a role for Shot in stabilizing the microtubule cytoskeleton through cross-linking to the actin cytoskeleton. Finally, through systematic deletion of potential motifs, we have determined the residues required for plus end tracking. These results demonstrate that Shot functions as an actin-dependent cross-linking molecule that utilizes microtubule dynamics for spatial and temporal targeting.

1059/B217
Microtubule Associated Enzymes That Determine Cell Shape.
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The cytoskeleton, which forms the structural backbone of all mammalian cells, is formed by several different networks of protein fibers. One of these networks is the microtubule (MT) network. MTs are tubes with a diameter of around 25 nm and a length that varies from 100 nm to over 100 μm. MTs are important determinants of cell shape and play an important role in cell migration, mitosis and long range intracellular transport. MTs have two distinct ends: the minus-end which is usually stabilized and anchored to the centrosome, an organelle localized near the nucleus of most cells. The other end is called the plus-end. This is the fast growing end of the MT and usually grows towards the cell periphery. MTs are very dynamic structures and different proteins play a role in the regulation of these dynamics. MT plus-end tracking proteins (+TIPs), proteins that associate with the growing ends of MTs, are important regulators of MT dynamics. So far, over 15 +TIPs with various functions have been identified. Some +TIPs are involved in the regulation of MT growth and shortage rates, some regulate the transition between phases of growth and shortage, again others serve to link MTs to other structures. We are studying a small family of +TIPs called navigators. Navigator proteins have been linked to the outgrowth and navigation of neurons. All members of the navigator family contain an AAA-type ATPase domain. Proteins containing this enzymatic domain have been linked to various activities such as proteolysis, membrane fusion and MT severing. To decipher the function of navigators and to examine the significance of their MT plus-end localization, we have analyzed the dynamic behavior of these proteins using fluorescent microscopy techniques. We have found that overexpression of navigator proteins in non-neuronal cells induces the formation of long cellular extensions. Interestingly, we discovered that this requires a functional MT binding domain and a functional ATPase domain. This suggests that navigators are enzymes that function at MT ends to facilitate cellular outgrowth.

1060/B218
Two MAP4 Isoforms Are Required for Muscle Cell Elongation and Fusion.
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During muscle differentiation, the microtubule cytoskeleton is reorganized from a radial pattern that emanates from the centrosome to a parallel array of microtubules that run along the length of the elongating myoblast. Previous studies have shown that the microtubule cytoskeleton is important for cell elongation, fusion and alignment of sarcomeric filaments in developing muscle fibers. We show that the parallel microtubule cytoskeleton in differentiated muscle cells is highly
stabilized with individual microtubules exhibiting reduced motility in comparison to undifferentiated cells. We think that structural microtubule associated proteins (MAPs) might be involved in crossbridging and stabilization of the microtubule network during myogenesis. We find that differentiating muscle cells express at least three major MAP4 isoforms. The expression of two isoforms, mMAP4 and oMAP4, is specific to myogenic differentiation, while the levels of uMAP4, the ubiquitous MAP4 isoform, remain unchanged in myogenesis. The two differentiation-specific isoforms differ from uMAP4 by two distinct insertions of ~120kD (mMAP4) and ~50kD (oMAP4) and we propose that these domains confer novel properties to the MAP4 molecules. Using RNAi and rescue constructs we demonstrate that oMAP4 is required for the efficient elongation and fusion of myoblasts during myogenic differentiation, while mMAP4 depletion positively affects cell elongation.

1061/B219
The Role of GgMAP215 in the Regulation of Microtubules in Axons and Growth Cones. 
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Axon dynamics are dependent on the precise, coordinated adjustment of cytoskeletal elements that underlie its morphology. Microtubules (MTs) are critical components of the cytoskeleton that serve as structural scaffolds and tracks for axonal transport. However, the mechanisms whereby these fibers are regulated during development are complex and poorly understood. MTs can be regulated through interactions with MT-associated proteins (MAPs). The members of the evolutionarily conserved MAP215/Dis1 family are well characterized in mitotic cells, important for MT stabilization, and essential for MT spindle formation. Objective: to test if GgMAP215, the chicken member of the MAP215/Dis1 family, is localized to areas of MT turnover in primary neurons. Materials and Methods: Primary chick peripheral neurons were dissociated and cultured for fixation and immunostaining. Immunocytochemical data was acquired using high resolution fluorescence microscopy and images were de-blurred offline using a constrained iterative deconvolution algorithm. Intensity correlation analysis (ICA, Qi et al. J.Neurosci 2004), a quantitative method for protein staining intensity covariance, was used to test for GgMAP215 MT association. A MT-disrupting drug, nocodazole, was used to depolymerize MTs and investigate whether GgMAP215 distributions were altered in the growth cone. Results: GgMAP215 puncta strongly stains the nodes of Ranvier, the initial segment, and the distal axon and growth cone, prominent areas of MT turn over in neurons. ICA analysis demonstrates that GgMAP215 and α-tubulin stains covary in de-myelinated axons, and suggests that after nocodazole treatment, GgMAP215 increases its association with reconstituting MTs during drug recovery. Conclusion: Our results localize GgMAP215 to sites of MT regulation in primary neurons indicating that this protein may play a key role in cytoskeletal dynamics.

1062/B220
Inhibition of Tau Filament Assembly by Peroxynitrite. 
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Tau is altered by numerous post-translational modifications during the progression of Alzheimer’s disease (AD). Some of these changes accelerate tau aggregation, while others are inhibitory. AD-associated inflammation is thought to create oxygen and nitrogen radicals such as peroxynitrite. In vitro, peroxynitrite is known to nitrate many proteins, including tau. We have previously demonstrated that tau’s ability to form filaments is profoundly affected by treatment with peroxynitrite and have attributed this inhibition to nitration of tyrosine residues. However, peroxynitrite is a highly reactive and unstable radical that not only nitrates tyrosine residues but also oxidizes amino acids through its radical byproducts. To test the possibility that peroxynitrite may also modify other amino acids via oxidative modifications, we constructed a mutant form of the tau protein lacking tyrosines (5XY→F). 5XY→F tau readily forms filaments; however, like wild
type tau its extent of polymerization was greatly reduced following peroxynitrite treatment. Since 5XY→F tau cannot be nitrated, it was clear that modifications other than tyrosine nitration are caused by the peroxynitrite reaction and that these modifications can change tau filament formation. We employed mass spectrometry analysis to identify non-nitrative alterations induced by peroxynitrite treatment of wild type tau and the 5XY→F tau. Peroxynitrite-treated wild type tau and 5XY→F tau consistently displayed lysine formylation throughout the tau sequence in an apparently random distribution. Lysine formylation likely results from reactive free radical exposure caused by peroxynitrite treatment. Therefore, our results indicate that peroxynitrite treatment of proteins In Vitro should not be used to study protein nitration as it likely induces numerous other random oxidative modifications clouding the interpretations of any functional consequences of tyrosine nitration.

1063/B221
The Chaperone DNAk Complexes with Tau and Inhibits Polymerization In Vitro.
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The microtubule-associated protein tau forms filamentous and non-filamentous aggregates which are associated with cognitive decline during the course of Alzheimer's disease and other tauopathies. Consequently, the prevention of tau's transition from a soluble, microtubule-bound state to insoluble aggregates is an important therapeutic target for several neurodegenerative diseases. Recently, we found an ~70 kDa protein contaminant in our histidine-tagged tau protein preparations that mass spectrometry revealed to be DnaK, the E. coli homologue of heat shock protein 70 (Hsp70). Hsp70 is a stress response chaperone protein that recognizes misfolded proteins and facilitates their degradation. Since the Hsp70 chaperone system plays an important role in the elimination of abnormal tau species, we were interested in determining whether chaperones such as DnaK and Hsp70 play a role in the prevention of tau aggregation via a direct interaction with tau. Given that we have always observed variability in the capacity for polymerization in our tau preparations, we hypothesized that this may be due to the presence of DnaK. First, we quantified the amount of DnaK present in several preparations and observed that the results are quite variable, ranging from 0.8 to 3.2 μM. Next, we determined that the addition of DnaK results in decreased tau polymerization; however, the overall effect depends on the tau preparation employed. Using ion-exchange chromatography, we successfully separated unbound tau from the tau-DnaK complexes. With the DnaK removed, we can now test the effect of Hsp70 on the polymerization of tau. Preliminary results demonstrate that Hsp70 inhibits tau polymerization. Our results suggest that molecular chaperones such as DnaK and Hsp70 directly inhibit tau-tau interactions that precede tau oligomerization and filament formation.

1064/B222
Structure-Function Analysis of Minispinules, the Drosophila Dis1/XMAP215 Homologue, Reveals a Necessary Region for Microtubule Lattice Association.
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Microtubule associated proteins (MAPs) are responsible for modulating the characteristic parameters of dynamic instability to accomplish essential cellular tasks such as kinetochore capture and organelle trafficking. within this class of cytoskeletal regulators, members of the Dis1/XMAP215 family act as microtubule polymerases, utilizing conserved TOG domains to bind tubulin heterodimers and accelerate microtubule growth by subunit addition at the plus end. While much attention has focused on the role of TOG domains in catalyzing microtubule growth, there is
little known about the molecular determinants that target Dis1/XMAP215 family members to microtubule structures such as the growing plus end, the microtubule lattice, and centrioles. To address this question, I performed a structure-function study of the Dis1/XMAP215 family member, Minispinelles (Msps) in Drosophila S2 cells. In interphase, wildtype Msp-GFP localizes to plus ends, is enriched along the lattice of select microtubules, and is found at inactive centrioles. By expressing fragments of Msps in S2 cells and assessing their localization, I have mapped a region of the protein necessary for microtubule lattice association. This interaction requires an intact TOG domain as well as a conserved ten amino-acid region found within the linker between two TOG domains. Targeted point mutations to either the linker region or the TOG domain that disrupt electrostatic interactions abrogate microtubule association of both the minimal microtubule-binding region as well as full length Msps. In addition, I have also tested the ability of these microtubule-binding mutants to rescue the cytoskeletal defects of Msps RNAi-depleted S2 cells. Together these data suggest that in conjunction with binding tubulin heterodimers through TOG domains, Dis1/XMAP215 family members have additional domains that facilitate their retention on the microtubule lattice.

**Microtubule Dynamics and Assembly I (1065 - 1076)**

**1065/B223**  
Polymerization of Tubulin, Antibody Coated Polystyrene Beads.  
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Microtubules have a key role in many biological functions in eukaryotic cells including providing a static and dynamic framework to maintain cell structure. In many single molecule experiments of microtubules/motor proteins where optical trapping techniques are implemented, antibody coated beads bind to either microtubule or motor proteins. Trapping and tracing the movements of attached beads lead to valuable information on static and dynamics of microtubules or motor proteins. In this study, instead of attempting to bind beads to preformed microtubules, antibody coated beads are polymerized with tubulins. Brightness and luminosity analysis of the primary images taken by DIC microscopy provides evidence of formation of single microtubules around antibody coated beads. Images indicate that beads are located inside microtubules. The formation of a microtubule-bead complex may offer a path to advance the study of microtubules inner structures.

**1066/B224**  
The Taccalonolides: Microtubule Stabilizers That Work through a Novel Mechanism of Action.  
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The taccalonolides are a novel class of microtubule stabilizers that do not bind to or affect the dynamics of purified tubulin or microtubules. Like all other microtubule stabilizers isolated to date, the taccalonolides cause microtubule bundling in interphase cells and mitotic arrest with multiple β-tubulin spindles. However, extensive biochemical analysis demonstrates that the taccalonolides do not bind to tubulin in the soluble or polymerized form and do not affect the polymerization of purified tubulin. This makes the taccalonolides the only microtubule stabilizers isolated to date that do not directly interact with tubulin. We are interested in elucidating the cellular biology of taccalonolide-dependent microtubule stabilization. Our current work demonstrates that a cytosolic extract supplemented with GTP and ATP is not sufficient for the taccalonolides to enhance tubulin polymerization in biochemical assays. The taccalonolides cause formation of extranumery β-tubulin spindles in mitosis through a mechanism that resembles fragmentation of the bipolar spindle pole. Interestingly, these cells can progress through an abnormal anaphase with up to six β-tubulin spindles. Normal centrosomal components, including gamma-tubulin, pericentrin and AuroraA, are absent from the majority of these β-tubulin spindles while other microtubule
associated proteins, including NuMA and Tpx2, colocalize with each spindle. We hypothesize that the taccalonolides stabilize cellular microtubules and form multipolar mitotic spindles in a unique manner from all other microtubule stabilizers isolated to date. Efforts to identify the taccalonolide binding site and mechanism of action will define a pharmacologically novel target of microtubule stabilization.

1067/B225

In Vitro Reconstitution of Microtubule Dynamics at Cell Cortex.

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The microtubule cytoskeleton is essential for fundamental processes such as mitosis, cell migration, axon outgrowth and neurotransmitter. Targeting of microtubules to specialized destination is critical for characteristic function and organization of microtubules. The microtubule-cortex interactions are presumably mediated by plus-end-tracking proteins (+TIPs) and cortex-associated proteins that bind to +TIPs. Although many of the factors involved have been identified, mechanistic understanding is still incomplete partly because of the complexity in cells. Here we reconstitute the microtubule-cortex interaction using mammalian +TIPs, EB1 and CLIP-170, and a cortex-associated protein IQGAP1 in vitro. Whereas EB1 autonomously recognizes growing ends, CLIP-170 does not end-track by itself and requires EB1 for its end-tracking in a manner similar to the previous report. In the microfabricated chamber, CLIP-170 comets are retained at and run along the IQGAP1-coated walls, whereas they disappear soon after they attach to the control walls. These results indicate that these +TIPs-decorated microtubules are navigated along IQGAP1. Thus, we propose the minimum machinery of microtubule guidance at cell cortex. Such In Vitro systems would be a powerful tool to analyze the mechanism of microtubule-cortex interaction and microtubule dynamics at the cortex.

1068/B226

Differential Stability of Plus and Minus Ends of Microtubules Revealed by Creation of Micro-Ablation Sites in Kinetochore Fibers Using a Focused Laser Beam.

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To understand the properties of microtubules (MTs) that make up kinetochore (K-) fibers, we directed a highly focused laser beam onto K-fibers to selectively ablate micro-domains and track the irradiation-induced structural damage with liquid crystal polarized light microscopy (LC-PolScope). We used crane-fly spermatoocytes with K-fibers that typically comprise 60 MTs during metaphase of the first meiotic division. By directing the focused laser pulses (20 to 500 pulses within a half second, each pulse 1 ns long with energy of 3 µJ at wavelength 532 nm, focused by 100x/1.3 NA objective lens) to the center or the edge of a K-fiber, a fraction of the fiber was severed leading to a micro-area of reduced birefringence (micro-ARB) of initially less than 1µm diameter, while maintaining continuity elsewhere in the fiber. Immediately following the ablation, on a time scale of seconds, we observed a very rapid loss of fiber birefringence in the direction towards the pole (rates of 12-17µm/min), while the birefringence in the direction towards the kinetochore remained stable. on a time scale of minutes, the kinetochore side of the micro-ARB moved poleward at a much slower rate (0.5-1.0µm/min) reestablishing K-fiber birefringence. After about 10 minutes, the K-fiber birefringence was virtually indistinguishable from the preoperational state and cells progressed through anaphase at a normal rate. Such operations were performed on the proximal (nearest to kinetochore) quarter of K-fibers during both metaphase and anaphase with similar results. We interpret the rapid loss of birefringence towards the pole to be a consequence of catastrophic depolymerization of microtubule plus ends, newly created by spot-ablation of kinetochore MTs. The much slower rate of K-fiber extension and repair is a
consequence of the poleward flux of microtubule polymer that is continuously assembled at kinetochores. Spindle dimensions, overall birefringence, and progression remained unaffected by these operations.

1069/B227
**Modeling FtsZ Polymerization Kinetics.**
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The bacterial tubulin homolog FtsZ can form single-stranded, linear polymers that assemble cooperatively. FtsZ has a sharp critical concentration and its polymerization kinetics can be fit by a model with a dimer nucleus. Previously, we and others have shown that linear polymers can have sharp critical concentrations if an unfavorable conformational change precedes the formation of stable polymers. However these previous models analyzed polymer thermodynamics and did not distinguish between several polymerization pathways, all of which can produce similarly sharp critical concentrations. Here we use computer simulations and mathematical analyses to determine which polymerization pathways can fit the concentration-dependence of FtsZ assembly kinetics. We find that while some pathways have dimer nuclei, as suggested by the original fits to the FtsZ data, several potential pathways have unimolecular nuclei instead. Models with unimolecular nuclei can in fact fit FtsZ polymerization kinetics under conditions where subunit activation and nucleation do not reach a rapid equilibrium. However in the models we have analyzed so far, unimolecular nucleation pathways require diffusion constants that are not biochemically realistic. In contrast, models with dimer nuclei can use biochemically reasonable rate constants. We are continuing to define which polymerization pathways are consistent with FtsZ’s assembly kinetics.

1070/B228
**Microtubule Organization and Dynamics in Skeletal Muscle: Microtubules under New Management?**
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Microtubules are typically nucleated at the centrosome, but several cell types, such as skeletal muscle, lack a typical centrosome. Some cell types also show microtubule nucleation at the Golgi complex, apart from that seen at the centrosome. In skeletal muscle, centrosomal proteins, microtubules, and Golgi complex are all redistributed, first during differentiation, and then further during maturation of muscle fibers. Differentiated muscle cultures show microtubules forming at the nuclear membrane but microtubule dynamics have never been explored in muscle fibers in vivo. Muscle fibers have a complex three-dimensional microtubule lattice. Understanding its organization would help us evaluate microtubule alterations detected in several muscle diseases. With this goal in mind, we have studied cold and nocodazole induced microtubule depolymerization and recovery in collagenase-dissociated fibers from the mouse flexor digitorum brevis muscle (FDB). After short recovery (15min) we observe numerous symmetrical microtubule asters centered on Golgi elements; most (85%) Golgi elements are involved. The asters subsequently release microtubules, which reorganize themselves longitudinally and transversely and after 24h, the recovered lattice is similar to that of control fibers. We have also examined the role of microtubules in the close association between Golgi elements and lysosomes (LAMP-1 positive structures) in muscle fibers. Most Golgi elements (75%) have juxtaposed lysosomes in control and nocodazole-treated fibers but cold and nocodazole, each, increase the fraction of Golgi-unassociated lysosomes. This suggests that both dynamic microtubules and stable, nocodazole-resistant, glutamylated microtubules are involved in Golgi-lysosome association. The proteins CLASP and/or GMAP-210 appear responsible for microtubule nucleation and/or anchoring at the Golgi complex in other cells. Immunostaining muscle fibers for CLASP has been inconclusive, so far. Our findings therefore provide the foundation of a model to understand microtubule and Golgi complex organization in muscle fibers. They also extend Golgi complex-based microtubule nucleation to a highly differentiated tissue. The mechanisms may not be identical.
1071/B229
The Molecular Mechanism of Cytoplasmic Linker Associated Protein (CLASP): Tubulin Dimer Binding Protein That Promotes Microtubule Rescues.
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Spatial regulation of microtubule (MT) dynamics by regulatory proteins is critical for cell polarity and cell division. Cytoplasmic Linker Associated Proteins (CLASPs) are a highly conserved class of microtubule associated proteins essential for mitosis and cell motility. CLASPs are critical MT stabilizers that promote MT rescues In Vivo and are localized in clusters on the MT lattice or at MT plus ends. Little is understood of the mechanism by which CLASPs contribute to these activities or whether they could function by themselves or in conjunction with other MT regulatory proteins. Using a combination of biophysical analysis, electron microscopy, we show that a recombinant full length S. pombe CLASP is a dimeric protein that binds a single tubulin dimer via conserved TOG-like domains. In Vitro analysis of dynamic MTs by total internal reflection fluorescence microscopy show CLASP increases MT rescue frequency, decreases MT catastrophe frequency and moderately decreases MT disassembly rate. CLASP binds the MT lattice in clusters while simultaneously binding tubulin dimers via TOG domains. Importantly, these clusters on the MT lattice correlate with sites of MT rescue events. Tubulin binding is critical for this activity, as TOG-L tubulin dimer binding inactivation disrupts CLASP activities, but not its MT lattice clustering In Vitro and in vivo. These studies provide a novel mechanism for MT rescue in which a group of CLASP molecules on the MT lattice reverses MT depolymerization through delivery of tubulin dimers.

1072/B230
A New Model Organism for Studying Microtubule Self-Organisation.
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The centrosome is widely recognised as the major microtubule nucleation site in animal somatic cells. However, microtubules are also generated independent of centrosomes in many cell types, and those microtubules play important roles in various intracellular processes such as mitotic spindle assembly or cell polarisation. With the aim of better understanding the mechanism of acentrosomal microtubule generation and organisation, we have started to utilise an emerging model plant, the moss Physcomitrella patens. We established a transgenic moss expressing GFP-tubulin and Histone-RFP, and applied time-lapse microscopy after drug treatment or RNAi knockdowns of microtubule regulators. We found that microtubules and F-actin are both critical for tip growth of chloronema and caulonema apical cells, two types of stem cells in the moss, and that microtubules, but not F-actin, are essential for spindle formation and chromosome segregation. Confocal imagings of mitosis in caulonema suggest that microtubules formed prior to nuclear envelope breakdown (NEBD) play a critical role in spindle assembly, and interestingly, a bipolar spindle is sometimes formed through conversion of the monopolar-like spindle that is assembled upon NEBD. We propose that P. patens could serve as a good model system to study acentrosomal microtubule self-organisation in cell growth and division.

1073/B231
Self-Organization of Acentrosomal Microtubule Arrays: Role of Arabidopsis Map65-1 in Microtubule Bundle Assembly and Dynamics.
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In eukaryotes, microtubule (MT) arrays provide a molecular framework for various cellular processes including cell morphogenesis, establishment of cell polarity and cell division. Powering
these cellular functions often hinges on the ability of the MT arrays to auto-organize in higher order structures. This is particularly true for non-centrosomal cells, such as plant cells where microtubule arrays do not emanate from a centrosome. Instead, they are mainly self-organized as MT bundles highly dispersed within the cell cortex. Thus, assembly of these cortical MT bundles is a key feature of cortical array organization and function in higher plant cells. How these MT bundles are generated and organized is still not well understood. Here, we study at the molecular level, MT bundle assembly and dynamics, by AtMAP65-1, the plant homologue of yeast Ase1p and mammals PRC1. This MAP cross-links MTs In Vitro, and is associated with interphase cortical MT bundles and the anaphase spindle midzone. Through studying biochemical mechanisms of AtMAP65-1 MT-binding and MT-bundling in vitro, we demonstrated that MAP65-1 promotes the bundling of anti-parallel aligned MTs and presented evidences for a mode of action that involves the binding of monomeric units to microtubules that “zipper up” microtubules through the homo-dimerization of their N-terminal halves when adjacent microtubules encounter. To further investigate the localization of AtMAP65-1 on MTs and its effects on MT dynamic parameters during MT assembly and MT bundle formation, we use an in Vitro assay based on single molecule TIRF microscopy. Experiments show that AtMAP65-1 is localized along and only with MT bundled and that its binding is concomitant with MT elongation. AtMAP65-1 does not significantly affects the speed of elongation or shortening of MTs within bundles, but MT inside AtMAP65-1-induced bundles usually do not depolymerize completely. As a result, the mass of MTs increases. Pause events are suppressed. These data demonstrate that MT bundling by AtMAP65-1 does not stabilized MT but bundles induce by AtMAP65-1 remain persistent over time and growth in vitro.

1074/B232 ABSTRACT WITHDRAWN

1075/B233

*Drosophila* Katanin Localizes to and Selectively Depolymerizes Microtubule Ends in Cells and In Vitro.

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Katanin is a phylogenetically conserved member of the AAA protein superfamily which removes tubulin subunits from the microtubule lattice thereby inducing internal breaks within the microtubule. Here we show that Katanin may also be selectively deployed to depolymerize microtubules from their ends. In particular, we have found that Drosophila katanin concentrates on the cell cortex and cortically positioned MT plus-ends in a variety of cell types. Its depletion by RNAi significantly reduces the frequency and rate of plus-end depolymerization and induces pronounced MT bundling at the cortex of interphase cells. These effects are rescued by co-depleting the +TIP, CLIP190. Finally, we have found that Drosophila katanin actively promotes slow microtubule end depolymerization, in vitro, and electron microscopy suggests that the mechanism by which this occurs is entirely distinct from that of kinesin-13s. We propose a model wherein Katanin selectively severs at or near microtubule plus-ends to remove regulatory “caps” which promote MT polymerization/stability.

1076/B234

Dissecting the Mechanism of Nuclear Migration in *S. cerevisiae*.

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In all eukaryotic cells, proper localization of the mitotic spindle is essential for precise chromosome segregation and inheritance of cell fate. Organization and localization of the yeast
mitotic spindle relies on cytoplasmic microtubules (MTs). MTs grow and shrink, and transitions between these phases occur via an apparently stochastic process called dynamic instability. Paradoxically, these stochastic transitions lead to a specific result, namely proper spindle positioning. How these fluctuations result in a stable, highly determined outcome remains unknown. We developed an integrated framework to automatically track and analyze the movements of MT plus ends and spindle poles in 3D movies. We investigated the effect of MT dynamics on spindle movement and how the regulation of MT dynamics contributes to proper spindle positioning. We show that MT dynamics are differentially regulated within the cell, and that this regulation impacts spindle movement. Our results indicate that the cleavage plane is critical in the control of MT dynamics, and that this regulation involves the septin-dependent kinases (SDKs) Hsl1p and Gin4p. Cells lacking these kinases have significantly altered MT dynamics and nuclear movement patterns. This indicates that SDKs control the functional behavior of yet undetermined MT-associated proteins around the cleavage plane confirming earlier observations from our group. In summary, we have developed a high resolution method to automatically track and analyze MTs and spindle poles in budding yeast cells allowing us to dissect the mechanism of spindle positioning in yeast cells.

Cilia and Flagella II (1077 – 1093)

1077/B235
Chondrocyte Mechanotransduction: A Role for the Primary Cilium.
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Chondrocytes, the only cell type in articular cartilage, have been shown to regulate the synthesis and degradation of a complex extracellular matrix in response to mechanical changes. Although it is clear that chondrocytes activate several signal transduction cascades in response to mechanical stimuli, how they convert mechanical signals into a biological response is poorly understood. This functional study aimed to directly test, for the first time, the hypothesis that chondrocyte primary cilia are required for the initiation of intracellular Ca\textsuperscript{2+} signalling pathways in response to mechanical compression. Wild type mice and Tg737\textit{orpk} mice lacking primary cilia were cross-bred with the ImmortoMouse and chondrocytes were extracted from the offspring to produce temperature-sensitive conditionally immortalized wild-type and \textit{orpk} chondrocyte cell lines. Cells were grown to confluence under permissive conditions and then were switched to non-permissive conditions for 72 hours prior to seeding in 3D agarose gels. Following 24 hours culture in serum, chondrocyte/agarose specimens were labelled with Fluo-4/AM and exposed to 20\% static compressive strain using a custom-made microscope-mounted compression rig. Changes in intracellular Ca\textsuperscript{2+} concentration were captured using wide field fluorescence microscopy, and characteristic Ca\textsuperscript{2+} transients were identified. The number of cells exhibiting unstimulated Ca\textsuperscript{2+} transients was significantly greater for uncompressed \textit{orpk} cells (46\%±8\%) than uncompressed wild-type cells (21\%±8\%), suggesting that cilia may be required for the regulation of spontaneous signalling. Compression caused a characteristic increase in the mean (±SEM) number of Ca\textsuperscript{2+} responses in wild-type cells (36\%±5\%), but a significant reduction in \textit{orpk} cells (26\%±3\%). The cilium-dependant compression response was significantly reduced in the presence of thapsigargin, but not EGTA, suggesting that the source of Ca\textsuperscript{2+} was related to external calcium rather than internal stores. Although this characteristic response to compression was not abolished completely in the absence of cilia, these functional data suggest that primary cilia are required in part for signal transduction in chondrocytes.

1078/B236
Tooth Germ Development Is Compromised in Cilia Mutants.
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In mammals cilia are nearly ubiquitous organelles that project from the surfaces of different cell types and are involved directly in many biological processes. Because of their ubiquity, defects in cilia structure or function lead to a wide range of developmental problems and diseases (ciliopathies) in humans. Ciliopathies are highly pleiotropic disorders, characterized by a multitude of symptoms, including abnormalities in dentition. Although cilia have previously been observed on odontogenic cells, their role in the formation of the mammalian dentition remains largely unknown. The goals of our study are to establish the spatio-temporal distribution of cilia on odontogenic cells in mice and to investigate their function in mammalian tooth formation using a conditional allele of the cilia gene \textit{Ift88} and tamoxifen inducible form of Cre recombinase. This approach will allow us to disrupt cilia and to investigate respective phenotypes at both the initiation and morphogenesis stages of tooth development. Using a combination of immunofluorescence assays and expression analyses of an \textit{Ift88} beta-galactosidase reporter allele we have demonstrated that most odontogenic cell types express cilia both at early stages of tooth development and at the stage of differentiation/secretion. Moreover, cilia can be detected on the cells of dental pulp in mature teeth, suggesting their role not only in tooth development, but also in the maintenance of tooth structure. To understand role of cilia in tooth germ development, we administered tamoxifen at E10.5, the day when tooth formation is initiated. Our preliminary data suggest that loss of cilia at this stage does not affect patterning of murine dentition. However, molar tooth germ development is severely compromised in \textit{Ift88} conditional mutants. Thus, at E15.5 the enamel organ does not form characteristic cap structure and is separated from underlying mesenchyme. Currently, we are analyzing changes in expression of signaling molecules and growth factors important for tooth development in cilia mutants.

1079/B237

\textbf{Cardiomyocyte Differentiation and Heart Development Is Coordinated by Stem Cell Primary Cilia.}

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Primary cilia are sensory organelles, whose defects in assembly or function are coupled to developmental defects and diseases in mammals. Here we looked into the role of the primary cilium in early cardiogenesis by investigating the function of the cilium in coordinating hedgehog (Hh) signaling and DMSO-induced differentiation of the pluripotent P19.CL6 stem cell line into cardiomyocytes. Further, we performed histological analysis on E11.5 day old \textit{Ift88} null (\textit{Ift88tm1Rpw}) embryos, which lack primary cilia, and conducted chimera analysis with cilia mutant and wild type ES cells to assess possible defects in heart development. We also determined the effect on cardiogenesis and expression of cardiomyocyte genes (Mef2C, Myh6, Myh7, Gata4, Nkx2-5 and α-actinin) in P19.CL6 cells when Hh signaling is inhibited by cyclopamine, a Smo specific antagonist, and by knocking down primary cilia using \textit{Ift88} and \textit{Ift20} siRNA. We show that P19.CL6 stem cells form primary cilia and that Hh signaling components such as Ptc-1, Smo and Gli2 localize to the cilia in these cells. Cyclopamine inhibits Hh signaling, preventing P19.CL6 cells from forming beating clusters of cardiomyocytes. \textit{Ift88} and \textit{Ift20} siRNA strongly reduced the formation of cilia, expression of mRNA and protein levels of cardiomyocyte markers, expression of Hh target genes, Ptc-1 and Gli1 as well as nuclear localization of Gli1. Furthermore, the induced loss of primary cilia with \textit{Ift88} and \textit{Ift20} siRNA strongly inhibited the formation of beating clusters of cardiomyocytes by maintaining the cells in their pluripotent state. In E11.5 day old \textit{Ift88} null embryos, we observed ventricular dilation, abnormal outflow tract development and abnormal myocardial trabeculae morphology compared to the wild type controls. In preliminary studies, chimera analysis indicates that ES cells lacking cilia are unable to
contribute to the atrial or ventricular chambers of the heart. These data support the conclusion that primary cilia are critical in stem cell maintenance and differentiation and in the regulation of early heart development, partly via coordination of Hh signaling.

1080/B238

Hedgehog Signaling Is Required for Gli3 Accumulation in Primary Cilia and Subsequent Degradation Independent of Processing.

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Hedgehog (Hh) signaling in vertebrates depends on intraflagellar transport (IFT) within primary cilia. The Hh receptor Patched is found in cilia in the absence of Hh and is replaced by Smoothened upon Hh stimulation. However, while the downstream transcription factor Gli3 localizes to cilia tips, it is unclear whether this process is Hh-regulated. Generating antibodies capable of detecting endogenous Gli3, we find unexpectedly that its ciliary localization requires active Hh signaling. Localization occurs within minutes of Hh addition, making it the fastest reported readout of pathway activity and permitting more precise temporal and spatial localization of Hh signaling events. We show that the species of Gli3 that accumulates at cilia tips is full-length, likely not PKA-phosphorylated and probably activated there into Gli3A. Moreover, by Western blot half-life determination, we show that Hh stimulation renders full-length Gli3 labile, leading to its proteasomal degradation via the nuclear SPOP/Cul3 complex, possibly following cytoplasmic microtubule-dependent transport. Furthermore, knockdown of IFT genes inhibits the degradation of full-length Gli3 as well as its processing, thus the Hh-induced lability of Gli3 likely contributes to the increased Gli3 activator:repressor ratios in IFT mutants.

1081/B239

Primary Ciliary Dyskinesia Protein 1 (Pcdp1) Is a Calmodulin Binding Protein That Localizes to the Central Microtubules and Is Required for Normal Ciliary Motility.

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Understanding how dynein is regulated to produce the complex waveforms typical of beating cilia is among the most pressing questions in the field. Numerous studies have shown that motility is modulated by changes in intracellular calcium, and that calmodulin (CaM) is a key calcium sensor associated with the axoneme. Using a CaM immunoprecipitation approach and *Chlamydomonas* axonemal extracts, we identified a complex that includes four polypeptides and specifically interacts with CaM in high [Ca$^{2+}$]. Using mass spectrometry we identified these polypeptides as FAP54, FAP46, FAP221, and FAP74 (flagellar proteome, Pazour et al, 2005). Based on sequence comparisons, FAP221 is orthologous to mammalian Pcdp1 (primary ciliary dyskinesia protein 1). Mice homozygous for a mutation in Pcdp1 suffer from primary ciliary dyskinesia and die soon after birth from severe hydrocephalus (Lee et al, 2008). Using a gel overlay assay we determined that a 58 amino acid region in FAP221 specifically binds CaM in high [Ca$^{2+}$]. Using the same overlay approach, we identified a calcium sensitive CaM binding site in mouse Pcdp1 which shares high sequence identity with the binding site in FAP221. To localize the Pcdp1 complex, we first took advantage of available *Chlamydomonas* mutants. The complex is lacking from or considerably reduced in axonemal extracts isolated from the central pair mutants *pf18* and *pf16*, but is present in extracts isolated from the *pf6* and *cpc1* mutants. All four proteins co-precipitate using anti-FAP74 antibodies and cosediment on sucrose gradients. Therefore, we predicted that they form a single complex that localizes to the C1c and/or C1d projections of the central pair. Since no mutation for any of these four proteins has been identified in *Chlamydomonas*, we knocked down expression of FAP74 using an RNAi approach. Thin-section EM of isolated axonemes revealed that these mutants lack the C1d projection. In addition flagella of mutants with reduced FAP74 expression are uncoordinated and have severely reduced beat frequency compared to wild-type. Based on these combined results, FAP221 is a CaM binding protein associated with the central microtubules and plays an important role in control of motility.
1082/B240
The Primary Cilium as a Negative Regulator of Canonical Wnt Signaling through Sequestration of Jouberin.
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Mechanisms of Wnt signaling regulation remain a fundamental question in biology. In particular, new insights into the role of the primary cilium as a regulator of both canonical and noncanonical Wnt signaling are being highlighted by disease mechanisms of ciliopathy pathogenesis. Here we identify a novel role for the ciliopathy disease protein Jouberin (Jbn) in canonical Wnt signaling. Jouberin localizes to the primary cilium yet its loss does not affect ciliogenesis In Vitro and In vivo. Jbn instead positively modulates canonical Wnt signaling through potentiation of nuclear β-catenin translocation. Further, Jbn is regulated by the primary cilium through sequestration in a manner dependent upon intraflagellar transport, leading to downstream inhibition of β-catenin nuclear translocation. Thus, the primary cilium is a negative regulator of canonical Wnt signaling at multiple points in the Wnt pathway.

1083/B241
Ciliary-Associated Hedgehog Signaling Is Altered in Polycystic Liver Disease.
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The epithelial cells lining intrahepatic bile ducts (i.e., cholangiocytes) express components of the Hedgehog (Hh) signaling pathway and are capable of releasing and responding to Hh ligands in cholestatic liver injury and cholangiocarcinoma. Activation of Hh signaling in these pathological conditions is linked to an increase in cholangiocyte proliferation. Hepatic cystogenesis in polycystic liver disease is also characterized by cholangiocyte hyperproliferation and structurally and functionally disrupted primary cilia. Because in many cell types Hh signaling strongly depends on primary cilia, our hypothesis was that in cystic cholangiocytes ciliary-associated Hh signaling is altered. Expression of components of the Hh signaling pathway in normal and cystic rat and human livers, and in cholangiocyte cilia of normal, PCK rats (an animal model of Autosomal Recessive Polycystic Kidney Disease, ARPKD) and patients with Autosomal Dominant Polycystic Kidney Disease (ADPKD) was assessed by RT-PCR, western blotting and immunofluorescence confocal microscopy. By RT-PCR, cholangiocytes of normal and PCK rats expressed message of all the major components of the Hh signaling pathway including: (i) the ligand, Sonic hedgehog (Shh); (ii) Hh receptors, patched (Ptc) and smoothened (Smo); and (iii) transcription factors, Gli-1 and Gli-2. Expression of Gli-2 was confirmed in normal and cystic cholangiocytes in culture by western blotting and in whole normal and PCK rat liver by immunofluorescence. Unlike their normal distribution (i.e., Ptc at the plasma membrane and Smo intracellular) in the liver of a patient with ADPKD, Ptc was localized by confocal microscopy to the apical plasma membrane and primary cilia of cholangiocytes lining liver cysts, whereas Smo was localized exclusively to the apical plasma membrane. In both, ARPKD and ADPKD, mRNA and protein of all components of the Hh signaling pathway were overexpressed in cholangiocytes. Thus, in cystic cholangiocytes, the ciliary-associated Hh signaling pathway is altered. These data suggest a role for Hh signaling in the benign hyperproliferation of the cholangiocytes in the polycystic liver disease.

1084/B242
Temporal Analysis of Primary Ciliary Function in the Pathogenesis of Polycystic Kidney Disease.
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Disruption of renal cilia results in renal failure characterized by tubular dilation and development of cysts. Using two independent conditional alleles of ciliogenic genes (KIF3A and Tg737/IFT88) and a tamoxifen-inducible CAGG-Cre line, we have disrupted cilia assembly at different stages in perinatal and postnatal life. Similar to that shown by Piontek et al 2007, our analysis shows that loss of cilia prior to P11 results in a rapidly progressing form of cystic disease, while induction after P12 leads to slow cyst development. These data indicate that more than simple loss of cilia mediated mechanosensation causes cyst formation. We hypothesize that a high proliferative environment typical of the perinatal kidney, combined with the loss of cilia, is required for the rapid cyst formation. To address this, we utilized transgenic mice constitutively expressing Cux-1. Cux-1 is a homeodomain protein which is highly expressed in nephrogenic zone of the developing kidney. It functions as a repressor of cyclin kinase inhibitors p21 and p27. Cux-1 transgenic mice develop renal hyperplasia and organomegaly without any structural defects and importantly do not develop cysts (Ledford et al. 2002). The increase in kidney size is correlated with high number of proliferating cells mainly in kidney cortex that continues into adulthood. To test our hypothesis, we generated tamoxifen-inducible IFT88 mutants on the Cux1 transgenic background and induced cilia loss at the age of P15 and P26. If cilia loss in a proliferative environment is responsible for the differences in the phenotype, we expect rapid cyst formation in the adult-induced cilia mutants. Interestingly, our data indicate that that loss of cilia in a high proliferative environment mediated by cux-1 is not sufficient to cause rapid cysts formation. This suggests the involvement of another mechanism, such as developmental reprogramming, that is needed along with ciliary dysfunction to induce the rapid form of cyst development.

1085/B243
CSPP Is a Ciliary Protein Interacting with Nephrocystin 8 and Required for Cilia Formation.
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The human ciliopathy Nephronophthisis (NPHP) is a monogenic cystic kidney disease, frequently involving retinal degeneration. To date, 11 disease loci have been identified (NPHP1-11). Mutations in these genes are thought to disrupt cilia related signaling pathways that affect proliferation and planar-cell-polarity in ciliated renal and retinal cells. Cilia assemble from the basal body that is formed by the mother centriole of the centrosome. We described earlier microtubule and cell cycle associated functions of two splice isoforms of the Centrosome Spindle Pole associated Protein, CSPP and CSPP-L, identified by us (Patzke et al., Oncogene 2005 and J Cell Physiol 2006). In the present study we investigated the expression and function of endogenous CSPP proteins with a newly developed antibody and identified the ciliary protein NPHP8/RPGRIP1L as an interacting protein. CSPP isoforms required their common C-terminal domain for this interaction and could form a ternary complex with both NPHP8/RPGRIP1L and NPHP4. Supportive for a physiological relevance of this interaction we found CSPP proteins to be expressed in ciliated renal and retinal cells In Vivo. In Vitro, endogenous CSPP isoforms localized to centrosomes and the midbody in cycling cell lines of epithelial and lymphoid origin. Interestingly, in ciliated epithelia cells CSPP proteins extended to the cilia axoneme. Moreover, RNA-i mediated depletion of CSPP proteins in the human diploid epithelia cell line hTERT-RPE-1 showed that they are required for primary cilia formation. Finally, similar to NPHP4 and its other interacting protein NPHP1 localization analysis in the polarized canine kidney epithelia cell line MDCK2 identified CSPP-L additionally at apical cell-cell junctions. To conclude, our investigation of CSPP expression at cellular and tissue levels identified a novel, non-mitotic function for CSPP isoforms. Their co-localization and interaction with proteins of a protein network at the primary cilium and cell-cell junctions that is crucial for normal renal and retinal tissue architecture and function together with the requirement of CSPP proteins for cilia formation define CSPP1 as a candidate gene for multi-organ phenotype ciliopathies.
The NPHP-Related Cystoproteins Form a Network That Regulates Primary Cilia Formation and Ciliary Signaling.

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Nephronophthisis (NPHP) and associated ciliopathies are autosomal recessive diseases characterized by kidney cysts, and often present with a range of extrarenal symptoms, including retinitis pigmentosa, cerebellar defects, and liver fibrosis. A number of the genes mutated in these diseases have been identified, and many of these gene products (cystoproteins) are localized to the primary cilia and centrosome, suggesting that alteration in ciliary signaling might be contributing to the progression of NPHP-associated diseases. To investigate how NPHP-related cystoproteins function in cilia tion and cystogenesis pathways, we have purified complexes associated with nine different cystoproteins from a fibroblast and/or an epithelial cell model using a recently reported system for high-throughput protein tagging and proteomic analysis (PMID: 19405035). We have identified a network of high confidence interaction proteins placing the NPHP-related cystoproteins in three sub-networks. In the first sub-network, NPHP1, NPHP4, and NPHP8 interact strongly among each other, and also bind to several actin-binding proteins. This suggests that NPHP proteins might be involved in pathways regulating the organization of actin cytoskeleton. In the second sub-network, NPHP6 binds to and directs NPHP5 to the centriole. NPHP5 also binds to NPHP2, which localizes to both centriole and primary cilium. NPHP5 is required for ciliation and might be important for organizing ciliary signaling around the centriole. In the third sub-network, NPHP6/CEP290 associates with MKS1 and MKS6, proteins participated in pathways important for neural tube closure and Hedgehog signaling. The identification of the NPHP protein networks provides a road map for further understanding the molecular mechanisms underlying ciliopathies.

Biochemical and Functional Evidence That the BBSome Functions as a Coat Complex in Ciliary Membrane Trafficking.

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The molecular etiology of Bardet-Biedl Syndrome (BBS), a retinopathy-obesity-nephropathy disorder, has so far remained elusive. A promising direction comes from our discovery of a stable complex of seven highly conserved BBS proteins, the BBSome, that we have implicated in vesicular trafficking to the cilium. To further our understanding of the molecular pathway that underlies BBS, we set out to functionally characterize the Arf-like GTPase Arl6 BBS3, a highly conserved BBS protein that is not part of the BBSome. The best characterized Arf-like GTPases, Arf1GTP and Sar1GTP, initiate COPI and COPII coat assembly, respectively, by recruiting coat complexes to donor membranes and subsequently enabling the coat complexes to capture transmembrane cargoes and bud a carrier vesicle. Interestingly, our structural analyses predict that the BBSome consists mostly of β-propeller and α-solenoid domains that form the core geometrical elements of COPI, COPII and clathrin coats. Most strikingly, affinity chromatography of retinal extract over Arl6GTP columns recovered nearly pure BBSome with near-quantitative yields. The interaction between Arl6GTP and the BBSome is extremely specific as Arl6GDP fails to capture the BBSome. Furthermore, shotgun mass spectrometry analysis of the eluates from the Arl6GTP and Arl6GDP columns indicates that the BBSome is the sole effector of Arl6 in retinal extracts. Using newly developed antibodies, we find that Arl6, like the BBSome, localizes to the primary cilium in mammalian cells, and siRNA-mediated gene replacement demonstrates that Arl6GTP is required to target the BBSome to the ciliary membrane. These data suggested that the Arl6 BBSome pair may function similarly to the Arf1/COPI and Sar1/COPII systems. Indeed, we find that Arl6GTP is necessary and sufficient to efficiently recruit highly purified BBSome to chemically defined liposomes. Finally, the ciliary membrane protein SSTR3 is mislocalized in mouse hippocampal neurons depleted of Arl6. Together, our data suggests that the BBSome
represents a novel coat complex akin to clathrin, COPI and COPII coats that mediates vesicular trafficking of ciliary proteins to, from or inside the primary cilium.

1088/B246
**Purification, Functional and Structural Studies of the Bbsome.**
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Bardet-Biedl syndrome (BBS) is a hereditary ciliopathy characterized by obesity, retinal degeneration and kidney disease. A genetically heterogeneous condition, BBS can be caused by mutations in at least fourteen different genes. Recently, seven of these BBS gene products, along with a novel protein, were found to form a stable protein complex that we named the BBSome. Cell biological studies, proteomic analysis and in silico structural predictions have led us to hypothesize that the BBSome functions as a protein coat that sorts transmembrane proteins to, from or within the cilium. In support of this hypothesis, we have discovered that the BBSome is an effector of the small GTPase Arl6 BBS3, a relative of the Arf family GTPases that regulate formation of COPI, COPII and clathrin protein coats. Problematically, our previously reported purification of the BBSome using tandem affinity purification yields only analytical (i.e. nanogram) amounts of non-homogenous material. To perform rigorous biochemical, functional and structural studies, we set out to establish a purification scheme of the BBSome from animal tissues. Retina and testis were chosen since their main cell types are known to have very high rates of ciliary trafficking. Through affinity chromatography onto Arl6GTP and polishing on MonoS, we are able to obtain sub-milligram quantities of nearly homogenous BBSome. This material allowed us to test for direct binding of candidate cargoes to the BBSome coat complex, and we find that a bacterially expressed intracellular domain of the leptin receptor (LepRbICD) can capture the purified BBSome in pull-down assays. Most strikingly, the direct interaction between LepRbICD and the BBSome is enhanced by Arl6GTP, reminiscent of the way in which Sar1GTP potentiates the interaction of the COPII coat with its cargoes. Embedding of the BBSome in vitreous ice and visualization by cryo-electron microscopy shows that the BBSome is monodisperse and is therefore a good candidate for 3D-reconstruction.

1089/B247
**Identification of Proteins Involved in G Protein-Coupled Receptor Ciliary Localization.**
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Bardet-Biedl syndrome (BBS) is a pleiotropic disorder that has been linked to dysfunction of primary cilia. We have found that disruption of BBS proteins affects cilia function through a mechanism that disrupts ciliary localization of G protein-coupled receptors (GPCRs). Interestingly, seven BBS proteins form a complex called the BBSome that localizes to the base of and within primary cilia. We hypothesize interactions between ciliary GPCRs and the BBSome are required for proper localization. To test this hypothesis we have used several biochemical approaches to identify the proteins mediating GPCR ciliary localization. Preliminary data suggest exciting putative ciliary GPCR interacting proteins, specifically an interaction through the GPCR i3 loop. From these results we will gain insight on the role of BBSome subunits and other proteins involved in GPCR ciliary localization.

1090/B248
**Dopamine Receptor 1 Localizes to Neuronal Primary Cilia in a Dynamic Process That Requires Bardet-Biedl Syndrome (BBS) Proteins.**
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Certain G protein-coupled receptors (GPCRs) localize to primary cilia on neurons throughout the brain. We have identified a ciliary localization consensus sequence in the third intracellular loop of ciliary GPCRs and used this sequence to identify dopamine receptor 1 (D1) as a novel ciliary GPCR. We further show that D1 ciliary localization is dynamic and D1 agonist treatment causes a rapid decrease in D1 ciliary localization. We previously reported that the BBS proteins are required for ciliary localization of GPCRs. Interestingly, we find that D1 ciliary localization is increased on BBS neurons and further show that agonist treatment does not reduce D1 ciliary localization suggesting that the BBS proteins are required for the translocation of D1 out of primary cilia. These results implicate BBS proteins in translocation of GPCRs both in to and out of the primary cilia.

**1091/B249**

**RPGR Is a Ciliary Protein Involved in Retinal Degeneration.**  
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Mutations in the Retinitis Pigmentosa GTPase Regulator (RPGR) are the most common single cause of retinitis pigmentosa. In photoreceptors, RPGR is localised in the connecting cilium, while the outer segment localisation is still under debate. These structures of the photoreceptor represent modified cilia, and, consequently, RPGR has also been found in the basal body and cilia of various ciliated cells and tissues. Furthermore, RPGR has been shown to co-immunoprecipitate with a number of axonemal or centrosomal Basal body proteins associated with microtubule transport. Available data suggest a role for RPGR in cilia formation and/or vesicle transport, but its exact function remains elusive. To gain further insight into the cellular role of RPGR, we analysed RPGR localisation and function in the hTERT-RPE1 (retinal pigmented epithelium) cell line. We confirmed that RPGR stains the cilia, whereas in non-ciliated cells the staining pattern is more diffuse throughout the cytoplasm. We also observed a difference in the migration of RPGR on Western blot in ciliated vs. non-ciliated cells, possibly due to translational modifications responsible for the ciliary targeting. Using siRNA-mediated depletion, we found that RPGR has a role in the early steps of cilia formation, but not in the later steps of cilia maintenance. Altogether, these data suggest that RPGR is involved in the process of ciliogenesis. Further studies will address the mechanisms of RPGR effect on ciliogenesis and how these can lead to retinitis pigmentosa.

**1092/B250**

**IFT88/Tg737 Is Essential for Perichondrial Architecture.**  
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The appendicular skeleton is patterned during embryogenesis and generated mainly by two cell lineages, chondrocytes and osteoblasts. The coordinated maturation and differentiation of the two cells types is necessary for correct growth and patterning of the skeletal elements. The role of primary cilia in the growth and development of the skeleton has only minimally been investigated and the requirement of the cilium on different cell populations within the developing bones remains unclear due to their expression on most cells throughout development. We have begun to analyze the requirement of cilia in the osteoblast lineage using a conditional mouse allele of the IFT gene, Ift88/Tg737. Ift88 is an essential component of the Intraflagellar Transport (IFT) particle and its disruption results in loss of the cilium axoneme. Previous work using the conditional allele of Ift88 and the prx1cre transgenic strain, which is expressed in the chondrocyte and osteoblast precursors of the developing limbs, showed that cilia are essential for endochondral bone formation and their disruption results in failure of bone collar formation and osteoblast differentiation in addition to defects in chondrocyte maturation and proliferation. To begin to analyze the role of the cilium specifically in osteoblast differentiation, we have begun to analyze conditional mutant mice with deletion of Ift88 in the osteoblast precursors without disrupting their expression in the cartilage (col1a13.6cre). In contrast to the results obtained using prx1cre, the bone collar of Ift88/coll1a13.6cre conditional mutant mice forms although the perichondrial architecture is disrupted and irregular. In microCT analyses, ossification of the
skeleton is delayed but does occur. The most striking phenotype observed in Ift88/col1a13.6cre conditional mutant embryos are defects in patterning of the long bones including incomplete ossification of the anlagen and ectopic mineralization in addition to changes in patterning of the long bones not limited to ectopic digit formation. We are beginning to investigate the pattern of osteoblast differentiation in the conditional mutant mice to determine what role the primary cilium plays in patterning of the skeleton.

1093/B251
Genetic Interactions of Chlamydomonas reinhardtii Eyespot Mutants.
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The eyespot apparatus is a photosensory organelle found in most flagellated green algae that allows the directional detection of light; the asymmetric positioning of the eyespot in the cell is crucial for generating proper phototactic responses. Cooperative interactions of cytoskeletal, chloroplast, and plasma membrane-localized components are essential for organizing and properly positioning this organelle. Genetic analysis of mutants of the single-celled green alga Chlamydomonas reinhardtii defective in either eyespot assembly or positioning has greatly contributed to our understanding of the mechanisms and components involved in defining the structure and subcellular localization of the eyespot apparatus. Two previously described mutants identified in a genetic screen include min1, possessing a miniature, equatorially-located eyespot, and mlt1, possessing multiple mis-positioned eyespots. The MIN1 gene encodes a C2-domain protein important for chloroplast envelope and plasma membrane apposition, while the product of the MLT1 gene has low sequence complexity and is not homologous to other known proteins. We hypothesize that MLT1 plays a role in cytoskeletal interactions that serve as positioning cues for the eyespot. Other genetic screens have yielded mutant strains in both miniature and multiple-eyespot phenotypic classes that are non-allelic to min1 and mlt1, but their genetic loci have not yet been identified. Phenotypic characterization of double mutants between unmapped eyespot mutants and min1 and mlt1, as well as the flagellar assembly mutant uni1, is suggestive that some eyespot proteins affect cytoskeletal and flagellar assembly. Characterization of such interactions in C. reinhardtii may provide a useful model for other systems wherein multiple interactions of membrane and cytoskeleton are involved in formation of complex subcellular structures.

Centrosomes I (1094 – 1110)

1094/B252
CentrioleDB: A Community Resource to Study Centriole-Derived Structures.
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Centrioles are cylindrical microtubule arrays required for stability and duplication of the centrosome in animal cells, and for the assembly of cilia and flagella in many eukaryotes. The great conservation of the centriolar structure and its broad distribution among eukaryotes suggest that centrioles were already present in the last eukaryotic common ancestor. In contrast to their core conserved structure, centrioles are assembled in a multiplicity of contexts, can perform several functions and display a diversity of accessory structures. Recent proteomic studies in human, green algae and ciliates have shown the conservation of a core group of proteins and highlighted that other components exist only in certain groups, likely reflecting the diversity discussed above. Genomic and ultrastructural data need to be integrated to understand centriole evolution, assembly and function. In particular it is very important to correlate molecules to morphological diversity. However, this diversity is not properly classified beyond model organisms. The information contained in decades of electronic microscopy of other organisms remains untapped. To address this issue, we created a bioinformatics tool that allows integrating
morphological information from microscopy images, detailed textual descriptions and molecular information. CentrioleDB, found at http://www.igc.pt/centrioledb, includes intuitive forms where users can upload and annotate images using a controlled vocabulary we developed, taxonomic information to help navigate and contextualize submitted images, orthologues for proteins of interest, and localization of proteins in centriole-associated structures. Currently, CentrioleDB contains hundreds of annotated images belonging to 25 different species as diverse as Chlamydomonas reinhardtii and Homo Sapiens, and protein information from about 38 organisms of interest. CentrioleDB, bridges the gap between morphological and molecular information. It allows the cell biology community interested in centriolar function and biogenesis to consider this structure in an evolutionary context. We anticipate that this database (centrioleDB) will catalyse a new dimension of cell biological research.

1095/B253
Regulation of Centrosome Size in the Early C. elegans Embryo.
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Organelle size is a crucial determinant of organelle and cell function and ultimately for survival. Consequently cells must have evolved mechanisms that can control and tune organelle size with respect to intrinsic biological changes. We set out to examine centrosome size throughout the development of the early c. elegans embryo. For this purpose, we have developed a fully automated system for tracking and measuring fluorescently labeled centrosomes in 3D time-lapse images. This algorithm enables us to analyze higher quantities of images with higher precision and reproducibility than would be possible by manual analysis. Using this system, we found that as the embryo undergoes sequential rounds of divisions, centrosomes scale with decreasing cell size. Reducing embryo size resulted in centrosomes of decreased size in all cell types of the embryo, indicating that centrosome scaling is independent of cell identity. By manipulating centrosome number, we further demonstrated that the total centrosome volume within a given cell appears to be the same as in the respective wild-type cell, indicating centrosome volume conservation. Importantly, this phenomenon not only holds true for within one cell type but in fact is also valid across rounds of divisions. We are currently screening among known centrosomal genes to identify regulators of centrosome size.

1096/B254
Involvement of CEP215/CDK5RAP2 in a Dynein-Mediated Transport of the Centrosome Proteins during Centrosome Maturation.
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The centrosome is an intracellular organelle acting as a major microtubule-organizing center (MTOC) in interphase cells. It also functions as a spindle pole in mitotic cells. Centrosome maturation is a process of expanding pericentriolar material (PCM) by accumulation of the PCM proteins prior to M phase. CEP215 is a human orthologue of Drosophila centrosomin, which is required for centrosome maturation, mitotic spindle assembly and development. Here, we characterized CEP215 as an essential PCM component. Depletion of CEP215 caused various defects in mitotic spindle assembly, which are reminiscent of those seen after dynein depletion. In fact, the centrosomal levels of dynein were reduced in CEP215-depleted cells. The dynein-mediated transport of PCM-1 and CG-NAP was also impaired. Our results suggest that CEP215 regulates a dynein-dependent transport of the PCM proteins during the centrosome maturation.

1097/B255
Characterization of the Centrosomal Ndr Kinases NdrA and NdrB in Dictyostelium discoideum.
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NDR (nuclear Dbf2-related) / LATS (large tumor suppressor) kinases are a subgroup of the AGC (protein kinase A/G/C -like) class of protein kinases. The genome of *Dictyostelium discoideum* encodes four NDR / LATS group kinases, NdrA-D. Sequence comparisons indicate that NdrA and NdrB belong to the NDR-like kinases while NdrC and NdrD are LATS-related kinases. In addition, three potential NDR activators of the Mob1 group, MobA-C, are found in *D. discoideum*. The human kinases NDR1 and 2 have been shown to play a role in centrosome duplication. In fission and budding yeast NDR kinases are involved in septation initiation and exit from mitosis, whereas the NDR kinase Orb6 in budding yeast is involved in the regulation of secretion. Here we explore the roles of NDR-related kinases in *D. discoideum* and describe a novel function of NDR kinases in regulation of phagocytosis. Live cell imaging showed that NdrA and NdrB localize to centrosomes, and NdrA was also present in isolated centrosome preparations. The localization of NdrA is regulated during the cell cycle. In prophase NdrA disappears from the centrosome and around the spindle forms a cloud-like structure, which is totally absent in later stages, until mitosis is completed. This change in localization is also observed for mutant NdrA bearing an altered hydrophobic motif, which mimics an inactivatable or phosphorylated state. Surprisingly, NdrA does not play a role in centrosome integrity or cytokinesis. Deletion of the *ndrA* gene results in reduced growth rates caused by defects in phagocytosis. This defect can be linked to vesicle transport by identifying the p24 family member EmpC as interactor of NdrA by immunoprecipitation. The NDR co-activators Mob1 of *D. discoideum*, MobA-C, show individual patterns of binding to the N-terminal Mob1 binding region of NdrA or NdrB In Vitro as compared to In Vivo studies. Whereas none of the Mob1 proteins could be found to bind NdrA in vivo, an In Vitro pulldown assay showed that MobA and MobB proteins are able to bind NdrA, suggesting that an activation step is required for the In Vivo interaction. In contrast to NdrA, NdrB was found to bind to MobB, In Vivo as well as in vitro, through a rather stable interaction.

1098/B256

**Plk4 Regulation and Centriole Formation in Xenopus Egg Extract.**

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Centrioles can organize two organelles in the cell - the centrosome and the cilium. Centrioles recruit pericentriolar material in dividing cells to form the centrosome and nucleate ciliary axonemes in both cycling and quiescent cell types in diverse organisms. Centrioles usually duplicate only once per cell cycle and only at sites near existing centrioles. In dividing cells centriole number is limited to ensure proper cell division, while in specialized multi-ciliated cells centrioles must be amplified to make the many basal bodies required. Centriole duplication is regulated by the activity of the Plk4 kinase. When Plk4 is overexpressed, multiple daughter centrioles form around existing mother centrioles, while in the absence of Plk4, no centrioles form. However, it is unclear how Plk4 activity is regulated and what proteins it modifies. To address these questions, we developed a system for assessing the centriole formation activity of Plk4 in *Xenopus* egg extract. We show that Plk4 overexpression causes both *de novo* centriole formation and amplification of added centrioles. Egg extract is useful for discovering interactions between centriole proteins since the concentrations of these proteins are much higher than in somatic cells. We are using several techniques to identify proteins interacting with Plk4 in our system. Surprisingly, we found that overexpression of a kinase-dead version of Plk4 also causes *de novo* centriole formation. A possible mechanism for this effect is that the mutant Plk4 titrates negative regulators that normally keep the endogenous protein in check. We hope to detail this mechanism of Plk4 regulation through structure/function studies and analysis of the endogenous Plk4 protein.

1099/B257

**Plk2 Phosphorylation Is Critical for CPAP Function in Procentriole Formation during the Centrosome Cycle.**

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Centrosome duplication is controlled in a tight link with the cell cycle progression. Genomic and genetic analyses have identified proteins that are required for centriole duplication in Caenorhabditis elegans. These proteins include SPD-2, ZYG-1 and SAS proteins (SAS-5, SAS-6 and SAS-4), all of which localize to centrioles. Recent studies suggest that the fundamental process of centriole duplication is evolutionally conserved. Here, we provide direct evidence that CPAP, a human homologue of SAS-4, is required for centriole duplication at G1/S phase. PLK2 was identified to be responsible for the phosphorylation at Ser589 and Ser595 residues of CPAP. Phosphorylation of CPAP is initiated at the procentriole during G1/S transition phase and it is diminished during M phase. Moreover, we revealed that this phosphorylation is important for CPAP function in centriole elongation. This work demonstrates an example that the biological activity of a centriolar protein is directly controlled by a protein kinase activity that oscillates during the cell cycle.

1100/B258
SAS-4 Is Required at Early Steps of Basal Body Duplication in Paramecium.
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Centrioles at the centrosome and basal bodies at the base of the cilia are two microtubule (MT) organizing centers that share the same architecture. They consist of a cylinder generally composed of nine MT triplets. Once per cell cycle, they duplicate following a common pattern. Several studies have shown that proteins conserved throughout evolution are involved in centriole and basal body duplication. The function of one of these proteins, Sas-4, has been recently characterized at the centrosome level in C. elegans, Drosophila and in vertebrate cell culture. EM tomography studies and epistatic analysis performed in C. elegans revealed that SAS-4 plays a role at the end of the assembly process, to allow the addition of MT singlets. Interestingly, however, the specific role of SAS-4 has never been investigated at the level of basal body duplication and during ciliogenesis. To answer to this question, we have used the multiciliated organism, Paramecium tetraurelia that contains around 4000 basal bodies. A really potent mechanism of gene inactivation by RNAi is available in Paramecium, allowing the detection of primary defects following the protein depletion. Using this approach, we could show that Paramecium SAS-4 is essential for basal body duplication, but is not required for cilia assembly. Following SAS-4 depletion we noticed that the majority of pre-existing basal bodies fail to duplicate and we did not detect any early structure such as germinative disc or cartwheel. In a few cases, when SAS-4 was only partially depleted, basal body assembly was initiated but was never accomplished, leading to the formation of intermediate structures presenting at the most nine MT doublets. Our results suggest that in Paramecium, basal body duplication is not a sequential process as previously described in centriole duplication in C. elegans. Instead, key proteins need to be present simultaneously to allow basal body assembly.

1101/B259
Dissecting the Roles of Centrin Isoforms in a Vertebrate Cell Line.
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In higher eukaryotes, successful cell division requires controlled microtubule polymerization and the correct assembly of the mitotic spindle. The centrosome constitutes the main microtubule organising centre and is responsible for the accurate segregation of chromosomes. Among the proteins associated with the centrosomes is a calcium-binding protein named Centrin. This small protein is highly conserved and unique to eukaryotic cells. There are several isoforms present in animal cells which have been reported to be involved in distinct pathways as nucleotide excision repair and mRNA/protein nuclear export. Previous studies using siRNA in human HeLa cells showed the requirement of Centrins for centrosome duplication and microtubule anchorage. However, a recent study using the human U2OS cell line showed conflicting results. Here we use
the hyper-recombinogenic chicken DT40 cell line to dissect the roles of the various Centrin isoforms. All 3 chicken isoforms, encoded by Cetn1, Cetn2 and Cetn3, are expressed in DT40 cells. We have successfully targeted all three Cetn loci, generating single, double and triple mutants of the Centrin genes in DT40 cells. RT-PCR, immunoblot and immunofluorescence microscopy have confirmed the absence of the Centrins. Unexpectedly, Centrin-deficient cells undergo normal cellular division with no obvious cell cycle defects. Light microscopy analysis of the cells did not reveal any significant difference in centrosome composition or the cellular microtubule network. Additionally, no perceptible abnormalities were found when analysing the centrosome ultrastructure using electron microscopy. However, we have observed a moderate increase in sensitivity to UV irradiation and 4-nitroquinoline 1-oxide (4-NQO) treatment in the Cetn2 null cells. Taken together, our results show that Centrins are not essential for centrosome duplication in DT40 cells but suggest their involvement in the repair of UV and 4-NQO-induced DNA lesions.

1102/B260
Reverse Genetic Analysis of Pericentrin.
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The centrosome is a subcellular organelle that arranges the mitotic spindle microtubules to ensure accurate segregation of chromosomes during cell division. Most animal centrosomes comprise a pair of centrioles which are encircled with an electron-dense substance termed the pericentriolar material (PCM). Pericentrin, which localizes to the PCM during the cell cycle, acts as a scaffold to recruit proteins including γ-tubulin to the centrosome. Mutations in the human pericentrin gene result in non-functional protein and dysfunctional cell cycle checkpoints. In this project we aim to define pericentrin functions in the cell cycle and in the response to DNA damage by reverse genetic analysis. We have generated Pericentrin (Pcnt) knockouts in the chicken DT40 model, using a strategy designed to target the promoter region of Pcnt. Targeting of the Pcnt promoter caused Pcnt mRNA levels to decline to 2% of wild-type levels, as determined by quantitative RT-PCR. No protein was detectable by immunofluorescence (IF) microscopy in the knockout cells. Pcnt-deficient cells are viable, but grow more slowly than controls. Consistent with the growth curve, the mitotic index of Pcnt-deficient cells was higher than wild-type cells, suggestive of a delay or arrest in the cell cycle at mitosis. IF microscopy showed that γ-tubulin, Centrin2, Centrin3, PCM-1 and Aurora a which bind to pericentrin, can localize to centrosomes in Pcnt-deficient cells, suggesting that centrosome structures are intact in these cells. Electron microscopy also confirmed that there were no significant abnormalities in the centrosomes of Pcnt-deficient cells. So far, the preliminary results suggest that loss of pericentrin did not disrupt centrosome composition.

1103/B261
Basal Body Duplication in Chlamydomonas.
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Despite much ongoing research, little is known about the duplication and assembly of basal bodies and centrioles. We have used Chlamydomonas as a model system to dissect the pathway of basal body duplication. Cells were synchronized by cycles of light and dark and prepared for electron tomography using high pressure freezing and freeze substitution. When shifted to the dark, the probasal bodies elongated to a mean length of ~380 nm. The first duplication intermediate was detected at this stage and consisted of a nine-spoked pinwheel structure that was positioned orthogonally at the face of the mature basal body, at prophase, the basal bodies separated into two complexes that migrated to opposite sides of the nucleus. Rootlet MT bundles remained associated with the two complexes and there was a noticeable increase in the number of cytoplasmic MTs. at metaphase, the nacent basal bodies were formed from a ring of 9 singlet MTs and were positioned at right angles to the mother basal bodies. Interestingly, the nacent
basal bodies were much longer than that detected at interphase with a mean length of ~210 nm and the pinwheel structure was also significantly elongated. The basal body complex did not appear to serve as the centrosome of the mitotic spindle but instead remained closely associated with the plasma membrane. Numerous kinetochore and nonkinetochore MTs were modeled and their pole-proximal ends were found to be distributed throughout the spindle. The length of probasal bodies at anaphase and telophase/cytokinesis was reduced (~170 nm) and doublet and triplet MT blades were detected. A combined approach, using mutants that lose control of these processes together with high-resolution 3-D structural studies, may give a deeper understanding of basal body duplication and assembly.

1104/B262
Characterization of the Human Centrosome Proteome Using Mass Spectrometry-Based Quantitative Proteomics.
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Objective Mass spectrometry-based proteomics have begun to address the protein composition of organelles and are also a powerful method for studies of changes in protein localization, modifications and interactions. However, it is necessary to be able to distinguish true components of subcellular structures from co-purifying contaminants. We have developed a quantitative mass spectrometry-based method to improve the correct assignment of the core proteome of subcellular structures and used it to obtain a detailed inventory of the human centrosome proteome. The list of centrosomal proteins provides the basis for spatio-temporal experiments as well as analysis of protein complexes that may help to better understand many of the functions associated with this organelle. Methods and results to enhance our ability to distinguish true components of subcellular structures, we developed a novel generic method based on protein correlation profiling (PCP) combined with Stable Isotope Labeling by Amino acids in Cell culture (SILAC). The method was used to characterize the human centrosome proteome and the obtained inventory comprises the majority of known centrosomal proteins and 57 new candidates, several of which were validated by microscopy. To study centrosomal import of newly synthesized proteins, we pulse-labeled human cells with stable isotope encoded lysine for 20 or 40 hours followed by centrosome isolation and analysis of isotope incorporation rates by mass spectrometry. Interestingly, we observed distinct incorporation rates for functional groups of proteins including the subunits of the γTuRC and HAUS complexes. To gain further insight into the function of the identified candidates, we used immunoprecipitation of GFP-tagged proteins and quantitative mass spectrometry to identify their interaction partners and to eliminate co-purifying false-positives. Conclusions We have performed a detailed characterization of the protein inventory of the human centrosome using a novel generic method, PCP-SILAC. Using this protein list, we have analyzed the centrosomal import of newly synthesized proteins as well as found interaction partners for selected centrosome candidate proteins.

1105/B263
Using DT40 Cells to Study the Function of the Centrosomal TACC Proteins.
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The Transforming Acidic Coiled-coil Containing (TACC) family of proteins are centrosomal proteins characterised by a highly conserved TACC domain at their C-terminus, which is essential for their centrosomal localisation. Three TACC proteins have been identified in mammals and
birds (TACC1, TACC2 and TACC3). We have previously shown that TACC3 co-operation with ch-TOG functions in formation of a bipolar spindle. However, the functions of the vertebrate TACC proteins and the functional relationship between them remains a mystery. To address this, we have are using the chicken B-cell line, DT40, in which targeted gene disruption is possible via homologous recombination. We removed the TACC domains of the chicken tacc1 and tacc2 genes. Cells without functional tacc1 and tacc2 genes are however viable and display no obvious centrosome or mitotic phenotype, indicating that tacc1 and tacc2 are dispensable in DT40 cells. In contrast, we were unable to generate tacc3 knockouts. This suggests that TACC3 may be essential for viability of the DT40 cells. Therefore, we are focusing our efforts to understand the function of TACC3 and its relationship with other centrosomal proteins. TACC3 phosphorylation by Ser/Thr kinase Aurora-A is required for TACC3 localisation in the centrosome. Whilst TACC3 is known to regulate microtubule dynamics at the centrosome, the function of TACC3 phosphorylation remains elusive. To determine the function of TACC3 phosphorylation, we have mutated the endogenous chicken tacc3 gene to a non-phosphorylatable or phosphomimetic form. Furthermore, we aim to systematically identify the interacting partners of TACC3 by tandem affinity purification (TAP) and mass spectrometry. for this purpose, we have TAP-tagged the endogenous chicken tacc3 gene. The interaction data will be used to determine the mechanism of TACC3 function in regulating spindle organisation.

1106/B264
Planarians Use the Acentriolar Pathway for Centriole Assembly but Lack the Centriolar Pathway.
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The centrioles forming the core of the animal centrosome and the basal bodies that nucleate ciliary axonemes in multiciliated epithelia are structurally very similar. They are however assembled following distinct pathways in animal cells. The duplication of the centrosome relies on the centriolar pathway, in which a single new centriole assembles in the vicinity of each pre-existing centriole. In contrast, in cells using the acentriolar pathway like differentiating multiciliated cells, multiple basal bodies assemble at one time around poorly defined structures called deuterosomes. We are studying the acentriolar pathway for centriole assembly using freshwater planarians as a model system. Planarians are flatworms that rely on a multiciliated ventral epithelium for locomotion, allowing direct observation of the phenotypes generated by RNAi against basal body proteins. We found that all the centriole genes identified in vertebrates are found in the genome of planarians and show high sequence similarity to their vertebrate counterparts. Disruption of these genes perturbed basal body assembly and inhibited ciliogenesis, suggesting that these proteins are also required for basal body assembly in the acentriolar pathway. Interestingly, inhibition of centriole Basal body genes did not affect cell division in regenerating planarians. We found that unlike other animal species planarians lack centriolar structures in proliferating cells and in most non-multiciliated differentiated cell types. Moreover, putative homologs of genes coding for components of the centrosome pericentriolar matrix were found neither in the planarian genome nor in the genome of another flatworm. Our results suggest that the typical animal centrosome structure was lost during the evolution of planarians. Thus, planarians and possibly all flatworms use the acentriolar pathway for centriole assembly extensively but appear to lack the centriolar pathway common to other animal species.

1107/B265
Dictyostelium Centrin B Interacts with Chromatin and the Nuclear Envelope.
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Centrins are small calcium binding proteins structurally related to calmodulins. Two centrin paralogs are found in Dictyostelium, DdCenA and DdCenB. Here we demonstrate by mutational analysis that the C-terminal globular domain (CGD) of DdCenB is essential and sufficient for
dynamic localization to the nuclear envelope (NE). Further truncation of the CGD into smaller fragments resulted in failure to localize to the NE. Removal of the C-terminus three amino acid extension or the KK duplet (both evolutionarily conserved features), or inactivation of the CGD putative SUMOylation site did not impair nuclear localization of DdCenB. However, substitution of the C-terminus of DdCenB by random sequence or a chimera to human centrins 1/2 resulted in incomplete delocalization from the NE at mitosis. Although the mechanism has not been elucidated yet, this indicates that the C-terminus of DdCenB plays a key role in the dynamic localization to the NE. To explore the role of DdCenB at the NE we first looked for interactions between DdCenB and chromatin. Interestingly, chromatin IP and co-IP assays indicate that DdCenB interacts (directly or through an unknown adapter protein) with DNA. Lack of DdCenB results in NE protrusions that extend to the centrosome and resemble those obtained after overexpression of DdSun1, a SUN domain protein involved in the anchoring of the centrosome connector to chromatin. In addition, 40 % of DdCenB null cells exhibited centromere cluster defects, as observed with DdCenP68. Since DdCenB is not particularly enriched at the centromere cluster, we developed a functional model where DdCenB is involved in the overall anchoring of chromatin to the NE. Lack of those anchoring points would affect centromere cluster organization and its interaction with the centrosome-to-nucleus connector, thus resulting in NE protrusions. This is consistent with the dynamic localization of DdCenB at the NE, whose disappearance at mitosis may be related to the release of chromosomes, allowing mitosis to proceed.

1108/B266
Dissecting the Centrosome Positioning Pathway.
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Cells, like whole organisms, have an incredible diversity of form. Cell patterning is achieved through the translation of polarizing cues into the specific arrangement of organelles and subcellular structures. Chief among these is the centrosome, the major microtubule organizing center of the cell. Despite being named for its central location in the cell, the centrosome can often occupy asymmetrical positions in differentiated cells, where it can contribute to the formation or function of cell structures such as cilia. During cell state transitions such as wound healing and polarization, centrosomes can also undergo transient repositioning within cells, and the function of this repositioning is not known. In the development of the *C. elegans* intestinal epithelia, centrosomes transiently shift from an anterior or posterior position in the cell to an orthogonal position at the future apical surface. This repositioning occurs at a developmental stage when cells are just beginning to form a polarized epithelium, and lack hallmarks such as apical junctions. We find that, following repositioning, centrosomal proteins become deposited along the apical surface of intestinal cells, suggesting that one function of centrosome repositioning might be to shuttle nucleators of microtubule assembly to the apical surface prior to polarization. In addition, we show that the polarity proteins PAR-3 and PAR-6 traffic with centrosomes to the future apical surface and are exploring the role of the centrosome in this localization. Finally, we are using visual genetic screens to identify genes that are involved in centrosome positioning in epithelial cells, and that link centrosomes to cell polarity.

1109/B267
TBCCD1, a New Centrosomal Protein Is Required for Centrosome and Golgi Apparatus Positioning.
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The mechanisms determining centrosome position in animal cells are not yet completely understood. Centrosome positioning is of obvious relevance for cytoplasm organization, cell polarity and migration. Geometrical constraints imposed by the substratum play a crucial role in centrosome positioning but probably do not provide the sole answer to this question. Current knowledge is that a dynamic radial microtubule (MT) network and the action of MT motors (e.g. dynein) are critical for centrosome positioning by exerting pulling/pushing forces on the cell cortex. So far few proteins were described as being important for centrosome positioning in mammalian cells. Here we show that TBCCD1 (TBCC-domain containing 1) is a new protein playing a pivotal role in centrosome positioning in close association with nucleus. TBCCD1 is a previously uncharacterized protein related with tubulin cofactor c (TBCC) and RP2 (retinitis pigmentosa 2). By the expression of GFP/RFP-TBCCD1 in human cells we showed that it localizes at the centrosome, the spindle midzone, midbody and basal body of primary cilia. The centrosomal localization of endogenous TBCCD1 was confirmed with a mouse polyclonal serum raised against GST-TBCCD1. The fact that TBCCD1 is essentially detected in the nuclear fraction, enriched in centrosomes, and that its centrosomal localization is independent of MTs, supports TBCCD1 as being a true centrosomal component. To test if TBCCD1, as RP2, has a functional overlap with TBCC a yeast complementation assay was performed in a ΔCIN2/TBCC strain with sensitivity to benomyl. The expression of TBCCD1 in the ΔCIN2 strain was unable to rescue this phenotype indicating that TBCCD1 may not have GTPase activating activity towards tubulin as TBCC and RP2. TBCCD1 silencing by RNAi in RPE-1 cells caused a cell cycle arrest in G1, a marked increase in the nucleus-centrosome distance, a disorganization of the Golgi apparatus, a decreased competence of primary cilia assembly and defects in directed cell migration. The function of TBCCD1 is under study in mammalian cells, and the function of this gene is also being analysed in the vertebrate model organism zebrafish, where we predict that it will have preponderant roles in development.

1110/B268  
Misregulation of Centrosome Positioning in Isolated Laminopathic Cells.  
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Cell polarity is a fundamental trait of living cells that involves intracellular reorganization of organelles, particularly the centrosome relative to the nucleus, in order to respond to both internal and external cues of an environment. Previous studies have reported that the centrosome is actively maintained at the cell center in confluent cells by proteins such as dynein, Par5, and PKC-zeta, while nuclear positioning is controlled by an independent pathway. Such studies suggest that cell-cell contacts are the primary polarizing cues that spur centrosome and nuclear centering. Cellular mechanisms responsible for polarization in isolated cells, however, are not yet fully understood. Using micropatterned substrates, we confined single cells to collagen islands of varying diameter and found that centrosomes were maintained at a position near the cell’s center. Isolated cells plated on unconfined substrates, however, displayed drastic centrosome-cell centroid distances. These results suggest that while cell-ECM contacts can serve as polarizing cues, centrosome centering is more strongly influenced by cellular confinement due to either nearby cells or a limited area of favorable substrate onto which a cell can adhere. Our previous results have shown that cells with nuclear lamin mutations, or laminopathic cells, fail to polarize in response to a shear flow stimulus. Here, using this single-cell polarization assay, we found that laminopathic cells displayed off-center centrosomes relative to wildtype cells. These results reinforce the essential role played by the LINC (Linker of Nucleus and Cytoskeleton) complex in mediating intercellular communication, specifically between nuclear lamins and the microtubule-connected centrosome, in order to coordinate cellular responses to external polarization cues.
Wiskott-Aldrich Syndrome (WAS) family proteins are Arp2/3 activators that mediate branched-actin network formation required for cytoskeletal remodeling, intracellular transport, and cell locomotion. WASP and Scar/WAVE, the two founding members of the family, are regulated by the GTPases Cdc42 and Rac, respectively. In contrast, linear actin nucleators, such as Spire and formins, are regulated by the GTPase Rho. We recently identified a third WAS family member, called Wash, with Arp2/3-mediated actin nucleation activity. We find that Drosophila Wash interacts genetically with Arp2/3, and also functions downstream of Rho1 with Spire and the formin Cappuccino to control actin and microtubule dynamics during Drosophila oogenesis. In addition to its actin nucleation activity, Wash bundles and crosslinks F-actin and microtubules. Wash is regulated by Rho1, Spire, and Arp2/3, and is essential for actin cytoskeleton organization in the egg chamber. Our results establish Wash and Rho as regulators of both linear- and branched-actin networks, and suggest an Arp2/3-mediated mechanism for how cells might coordinately regulate these structures.

PKA (Protein Kinase A) is involved in many physiological phenomena, including sugar metabolism and ion channel control, as well as cell-migration. PKA needs to form a complex with AKAP(A Kinase Anchoring Protein) to phosphorylate specific substrates, but there are few studies of AKAPs associating with cell migration. WAVE2 (Wiskott-Aldrich syndrome protein) is a key regulator, which enhances the lamellipodia formation by promoting Arp2/3 dependent actin polymerization. Our previous study showed that PKA can regulate the expression of WAVE2 and that PKA may associate with WAVE2. To reveal the function of PKA as a regulator of cell-migration, we examine the interaction between PKA and WAVE2 in detail. First, we performed reciprocal co-immunoprecipitation assay and found that exogenously expressed PKA was co-precipitated with WAVE2 in COS-7 cells. Endogenous PKA was also co-immunoprecipitated with WAVE2 in mouse brain extract. PKA catalytic subunit dissociated from the WAVE2-PKA complex by the addition of 8Br-cAMP in vitro. These results suggest that the complex includes PKA holoenzyme. WAVE2 deletion mutants revealed that the WHD and VCA region of WAVE2 are involved in the complex formation. Immunofluorescence assay showed that PKA and WAVE2 co-localized at lamellipodia in MDA-MB-231 cells. We next examined the effect of PKA activator or inhibitor on lamellipodia formation by using time-lapse microscopy. PKA activation by Forskolin/IBMX enlarged lamellipodia and PKA inhibition by H89 retracted membrane protrusion. Knockdown of WAVE2 by siRNA inhibited the lamellipodia elongation induced by PKA activation, whereas it did not affect lamellipodia formation without PKA activation. In conclusion, PKA forms a complex with WAVE2 at membrane ruffles and controls membrane protrusions through WAVE2. These results indicate that WAVE2 may function as AKAP associating with lamellipodia formation and cell migration.
The small Rho GTPases, Rho and Cdc42, are key regulators of the cytoskeleton and organize biological activities in numerous cellular processes including wound healing, cytokinesis, and morphogenesis. During single cell wound healing, Rho and Cdc42 form discrete zones of activity around the wound perimeter, where Rho activity is primarily confined to the wound edge and is circumscribed by a zone of Cdc42 activity. These concentric zones of GTPase activity move forward with remarkable precision that mirrors the movement of the actomyosin ring they template. It is unclear how this precise forward movement is accomplished. Using a combination of photoactivation and molecular manipulation approaches we show that the apparent movement of the GTPase zones is illusory, and in fact the zones move via a “Signal Treadmill”. In the treadmill, the GTPases are preferentially inactivated at their trailing edge, and the rate of inactivation is entrained to the rate of contractile array movement. Further, suppression of Myosin-2 powered flow results in disorganization of the treadmill. These results indicate that differential flux through the GTPase cycle imparts directionality on the contractile arrays. They also suggest that one of the major roles of contractility is to control the organization of the treadmill.

1114/B272  
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Nuclear movement during centrosome orientation in fibroblast occurs by coupling the nucleus to retrogradely flowing dorsal actin cables. The origin of the actin cables that move the nucleus and the factors involved in their organization are unclear. We tested whether the nucleus itself might contribute to the formation or organization of the actin cables that stimulate/power its movement. We found that expression of factors known to stimulate nuclear movement in 3T3 fibroblasts (Q61LCdc42 or MRCK), led to a pulse of myosin activity (measured by pSer19MLC immunofluorescence) and the formation of actin cables near the nucleus before cell-wide myosin activation, suggesting that the nucleus may specify its own actin/myosin network. To further test this, we depleted nuclear envelope proteins including emerin, nesprin2G, lamin A/C, and lamin B1 by siRNA and found that only depletion of emerin blocked the pulse of pSer19MLC near the nucleus. Furthermore, emerin was detected on the outer nuclear envelope and coimmunoprecipitated with pSer19MLC and myosinIIIB but not myosinIIA. Depletion of emerin, myosinIIA, and myosinIIIB blocked rearward positioning of the nucleus but by distinct mechanisms. MyosinIIA depletion completely blocked nuclear movement and resulted in centrally positioned nuclei. Depletion of emerin or myosinIIIB caused random rather than directed rearward nuclear movement and resulted in nuclei dispersed throughout the cytoplasm. Live cell imaging of actin revealed that depletion of myosinIIA inhibited the formation of dorsal actin cables, whereas depletion of myosinIIIB or emerin inhibited the formation of the ventral actin bundles under the nucleus. Although the dorsal actin cables formed in emerin and myosinIIIB depleted cells, their flow was not directed retrogradely from the leading edge; instead, the dorsal actin cables either flowed toward the cell center from all sides or swirled around the periphery of the cell. These data suggest a model where the nucleus via emerin sets up a myosinIIIB dependent contractile system at the cell center which provides the proper directionality to the myosinIIA generated dorsal actin cables that can then move the nucleus rearward.

1115/B273  
Dynamics of Reconstituted Active Actin Networks.  
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Cells have to adapt their local mechanical and structural properties in order to fulfill their physiological tasks. While already purely crosslinked networks can show clear out of equilibrium properties, the activities of molecular motors ensure the local adaptability of the cytoskeleton. Therefore, we study the effect of molecular motors in crosslinked networks using fluorescence
microscopy. Dramatic structural rearrangements appear in an actin/fascin network in the presence of Myosin II minifilaments. These can be analyzed using a correlation algorithm. It appears that a sensitive balance of time scales for unbinding of crosslinkers and motor activity are the clue for the understanding of such complex active networks.

1116/B274
Roles of Actin-Based Protrusions in Cell-Cell Junction Formation in Endothelial Cells.
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Compartmentalization and integrity of tissues depends upon coherent cellular sheets possessing extensive cell-cell junctions. Belt-like adherens junctions that are mediated by cadherins and strengthened by the actin cytoskeleton are a key element of these interactions. The procedure by which cells establish continuous adherens junctions de novo remains largely mysterious. Observations of cell-cell contact formation in various epithelial cells suggests two models, one of which commences with filopodia that extend from adjacent cells and establish a series of initial point contacts, which subsequently zipper into a continuous cell-cell junction. Another model proposes that lamellipodia establish the initial junction, while subsequent contact-dependent inhibition of protrusion in both cells produces stable contacts. It remains unclear whether these models are mutually exclusive and whether either of them is applicable to other cell types. We investigated the dynamics of cell-cell junction formation and the corresponding architecture of the underlying cytoskeleton in cultured HUVECs. Our data show that free cell edges express lamellipodia undergoing protrusion-retraction cycles and that the initial interaction between cells is mediated by protruding lamellipodia. As revealed by platinum replica electron microscopy (EM), these lamellipodia contain typical branched actin filament network with very few embedded actin bundles characteristic for filopodia. However, upon subsequent retraction of contacting lamellipodia, interdigitating filopodia-like protrusions connecting adjacent cells become obvious. They emerge from one or both cells and are bound to each other or to the lamella of the adjacent cell by VE-cadherin-rich junctions. EM showed that these protrusions contain a tight bundle of long actin filaments suggesting that they are similar to conventional filopodia. We propose that there is a possible functional relationship between initial point contacts made by lamellipodia and initiation of filopodia at these sites, while induced filopodia may strengthen the nascent junction and maintain the cells close to each other, which would increase chances of junction expansion. Supported by NIH grant GM 070898.

1117/B275
Filopodia Formation on Exposure to Hypertonic Stress Is Regulated by Hsp27 and P38 MAPK in Acute Myelogenous Leukemic Cell Line Kg1a.

Cells address different stresses using different mechanisms to regain cellular homeostasis and to protect stressed cells from apoptosis (Alfieri and Petronini, 2007). The human acute myelogenous leukemic cell line, KG1a extrudes filopodia in medium with increasing salt concentrations (Oh et al, 2000). When osmolality of isotonic media is increased from 255 mOsm to 745 mOsm, more than 90% cells show filopodia at the end of 1 hour of exposure to osmotic stress. In order to elucidate the molecular mechanism regulating the filopodia formation, we investigated the expression of heat shock protein 27 and p38 MAP kinase by real time PCR and western blot analysis, since both hsp27 and p38 have been implicated in cytoskeletal changes in various studies. Both hsp27 and p38 were found to be up regulated more than two fold on exposure to hypertonic stress. Treatment with KNK 437, a known inhibitor of heat shock proteins showed drastic reduction in filopodia forming KG1a cells under hypertonic stress and also showed comparable drop in levels of HSP27 mRNA. Western blot analysis indicated reduction in hsp27 protein under similar conditions. SB203580, a p38 MARK inhibitor, markedly reduced filopodia formation and caused a drop in p38 mRNA levels, which was shown by real time PCR analysis.
In order to ascertain the cytoskeletal composition of the hypertonic stress induced filopodia, fluorescent staining of cytoskeletal components was done which suggested the involvement of actin and tubulin in these filopodia. KG1a cell treatment with microtubule inhibitors resulted in an expected decrease in the percentage of filopodia-forming cells, indicating an important role played by microtubules in filopodia formation. On the contrary, exposure of KG1a cells to the actin depolymerizing agent, Cytochalasin D, enhanced the formation of filopodia when the cells were maintained at 255 mOsm and did not inhibit the formation of filopodia at 745 mOsm. Cytochalasin D seems to mimick hypertonic stress by stimulating the filopodia formation. Whether the induction of filopodia occurs by the same p38-hsp27 mediated pathway, is currently being investigated.

1118/B276
Mice with Tropomodulin1-Deficient Red Blood Cells Exhibit Increased Levels of Tropomodulin3 and a Mild Hemolytic Anemia.
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The shape and deformability of red blood cells (RBCs) are determined by a membrane skeleton, comprised of a network of short actin filaments that are cross-linked by long, flexible spectrin tetramers. Strikingly, RBC actin filaments are tightly maintained at a length of ~40 nm. A priori, the polymerization and stability of these actin filaments is expected to be critical for the network's long-range connectivity and mechanical function. RBC actin filaments are normally capped at their pointed ends by tropomodulin1 (Tmod1) and stabilized by tropomyosin (TM). However, direct evidence is lacking regarding the importance of pointed-end capping by Tmod1 in RBC membrane skeleton biogenesis, organization, and stability. Therefore, we used a Tmod1-knockout mouse model to investigate the role of Tmod1 in the RBC membrane skeleton. Western blots of Tmod1-deficient RBC membranes confirm the complete absence of Tmod1 but also reveal an unexpected compensatory increase in tropomodulin3 (Tmod3), an isoform that is normally not present in RBCs. TM levels are also slightly reduced, but levels of spectrin, adducin, protein 4.1 and other RBC membrane skeleton proteins appear unchanged. Hematological analyses reveal that Tmod1-knockout mice have a compensated mild hemolytic anemia, with RBCs that are abnormally variable in size and hypochromic in blood smears. Osmotic fragility and ektacytometry measurements indicate that Tmod1-deficient RBCs are more mechanically fragile and less deformable than wild-type RBCs. Thus, compensatory upregulation of Tmod3 does not completely ameliorate the pathological characteristics of Tmod1-deficient RBCs. This is likely due to insufficient amounts and altered biochemical properties of Tmod3, which, unlike Tmod1, can bind actin monomers and nucleate actin filament assembly in addition to capping pointed ends. We propose that Tmod3-capped actin filaments are more dynamic, with more variable lengths that lead to instability in the membrane skeleton of circulating RBCs.

1119/B277
Actin Cytoskeleton Remodeling Is Regulated by the Rho GAP DLC1 during Nucleotide Depletion.
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Ischemia causes a depletion of cellular nucleotides and remodeling of the actin cytoskeleton in kidney proximal tubule cells. We previously showed that a loss of RhoA activity is responsible for the decrease of stress fibers following nucleotide depletion. However, the mechanism of RhoA activity regulation during ischemia is unknown. We therefore decided to look for RhoA regulators of importance during ischemia. Deleted in Liver Cancer 1 (DLC1) is primarily localized to focal adhesions. It is a Rho GAP with activity for RhoA, B and C. We found that knockdown of DLC1 with siRNA contributed to the preservation of stress fibers during antimycin a induced nucleotide depletion of cultured mouse kidney S3 proximal tubule cells. In addition, using a RhoA pull-down assay, we found RhoA activity was also preserved during nucleotide depletion after DLC1 knockdown. DLC1 is known to shuttle in and out of the nucleus. We confirmed this movement in
S3 cells that were transiently transfected with GFP-DLC1. Treatment with a nuclear export inhibitor resulted in an accumulation of GFP-DLC1 in the nucleus of S3 cells. Cell fractionation experiments confirmed that DLC1 accumulates in the nucleus during nucleotide depletion. During ischemia the ATP/AMP ratio decreases and AMP-dependent kinase (AMPK) is activated. Using two different compounds that activate AMPK, metformin and resveratrol, we found DLC1 moved into the detergent insoluble (nuclear) fraction. These results confirm that DLC1 activity is required for maximal RhoA inactivation and cytoskeletal remodeling during ATP depletion, that DLC1 localization is altered in response to ATP depletion and suggests an upstream regulator through which these events may occur.

1120/B278
AZX100 Modulates Actin Dynamics in Hypertrophic and Keloid Myofibroblasts.
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Keloid and hypertrophic scar-derived myofibroblasts are characterized by increased stress fibers and contractile function as compared to fibroblasts. This is demonstrated as an increased ratio of filamentous (F) actin to globular (G) actin. Previous research suggests that AZX100, a 24 amino acid phosphopeptide analogue of Heat Shock Protein Beta 6 (HSPB6 or HSP20) containing a protein transduction domain, binds to 14-3-3, displacing phospho-cofilin and increasing cofilin-dependent depolymerization of actin, resulting in an increased pool of G-actin. As a result, the ability of AZX100 to reduce the F-actin content of dermal myofibroblasts was investigated in this study. Experimentally, keloid and hypertrophic scar-derived fibroblasts were serum starved for 24 hours, followed by treatment with Transforming Growth Factor Beta 1 (TGFβ1) at 2.5ng/ml alone or in the presence of 25μM AZX100 for 24 hours. F-actin was then separated from G-actin by ultra centrifugation techniques and the amounts of each were determined by Western blot analysis. AZX100 treatment of hypertrophic and keloid derived myofibroblasts decreased the pool of F-actin by 20% and 16%, respectively. AZX100 treatment of keloid and hypertrophic myofibroblasts visually reduced stress fiber formation as measured by immunofluorescence. Additionally, treatment of fibroblasts with AZX100 in the presence of TGFβ1 reduced the amount of α smooth muscle actin mRNA as compared to fibroblasts treated with TGFβ1 alone. The data suggests that AZX100 disrupts the cytoskeleton network in persistent myofibroblasts and improves healing by decreasing fibrotic scar formation.

1121/B279
Contributions of Zyxin and Mena/VASP to Stretch Induced Actin Stress Fiber Reinforcement.
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Organs such as lung, heart, and the vasculature experience regular mechanical stimulation, but our understanding of cellular and molecular mechanotransduction remains incomplete. Integrin-rich focal adhesions may sense, transmit and respond to mechanical input, and contain several mechanosensitive molecules. Zyxin is a LIM-containing adaptor protein prominent in mechanically stimulated tissues, it accumulates at integrin-rich cell-matrix adhesions and is acutely sensitive to mechanical stimuli, such as uniaxial cyclic stretch applied to cells in culture. We previously reported that zyxin’s predominant subcellular distribution at focal adhesions shifts to actin stress fibers upon stretch, and that stretch-stimulated stress fiber thickening is abrogated in fibroblasts derived from zyxin-null mice. Mammalian enabled (Mena) and Vasodilator Stimulated Phosphoprotein (VASP) bind to four proline-rich ActA repeats in zyxin and also exhibit stretch-stimulated actin stress fiber accumulation. We hypothesized that Mena/VASP binding to zyxin contributes to stretch-induced stress fiber thickening. We disrupted the zyxin-VASP interaction by mutating zyxin’s ActA repeats, changing four critical Phenylalanine residues to Alanine (zyx4F>A). Wild-type zyxin and the zyx4F>A mutants were expressed in zyx-/- fibroblasts and the stretch responses were evaluated by phalloidin-staining of F-actin and zyxin and Mena/VASP localization by fluorescence microscopy. Stress fibers in cells exposed to uniaxial
cyclic stretch thickened with reintroduction of wild-type zyxin, which distributed along the actin filaments. Mena/VASP co-distributed with wild-type zyxin along the actin filaments but did not accumulate with zyx4F>A mutant along the stretch-stimulated actin filaments. Disruption of Mena/VASP binding to zyxin significantly attenuated stretch-induced stress fiber thickening even though the zyx4F>A accumulated along those actin stress fibers. These data indicate that zyxin recruits Mena/VASP to stress fibers in response to mechanical stimulation and that Mena/VASP contributes to the stress fiber thickening.

1122/B280
Numerical Simulation of Force Distribution in Cytoskeleton during Cell Spreading.
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The shape, motility and biological functions of cells are much affected by cytoskeleton. Most cell behaviors start from a spreading state instead of a round state; therefore, an applicable cytoskeleton model with a certain degree of attachment is essential for studying more cell behaviors. Tensegrity is utilized as a numerical cytoskeleton model to simulate cell spreading and investigate the changes of mechanical properties during cell spreading. Tensegrity with compression-tension system is verified to have consistent features with cells. In our study, we use tensegrity to simulate cell spreading to find a cell-like cytoskeleton deformation. The change of force distribution within tensegrity during cell spreading was also observed from each tensegrity elements. The cell contractile force during cell spreading will be also investigated. By finding deformed tensegrity corresponding to different degree of cell attachment, the contractile force and stored energy of cytoskeleton are found to increase with the increasing attached area. Further, the energy of cytoskeleton is more contributed by filaments than microtubules, especially for a large spreading. The force distribution of the attached nodes is also consistent with observations in cells. Therefore, tensegrity is verified to have potential to analyze more cell behaviors associated with a spreading state.

1123/B281
A 68 Amino Acid Insert Changes an Actin Bundling Protein into an Actin Severing Protein.
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Vinculin and its splice variant metavinculin are actin binding proteins involved in the formation of large macromolecular assemblies such as podosomes and invadopodia, adhesion junctions, dense plaques, intercalated disks and costameres. While vinculin is expressed ubiquitously, metavinculin is mainly expressed in muscle tissue, where metavinculin can form up to about 50% of the total vinculin pool (Belkin et al., 1988). Vinculin and metavinculin are identical except for a 68aa acidic insert in the C-terminal domain (tail domain) of metavinculin. for both isoforms, the tail domain contains the actin binding site. By using transmission electron microscopy we determined at high resolution how vinculin (Janssen et al., 2006) and metavinculin (this poster) organize actin filaments. Although both isoforms bind actin filaments in a similar way, interestingly enough they organize actin filaments in a completely different manner. In addition to actin-binding, vinculin bundles actin filaments into a high order assembly. Our in-vitro studies show that metavinculin, in addition to filament binding, severs actin filaments and prevents vinculin from forming actin bundles. We are using biophysical, biochemical and microscopy techniques to determine the mechanism by which metavinculin co-operates with vinculin in actin organization. References: - Belkin, A. M., et al. Immunolocalization of meta-vinculin in human smooth and cardiac muscles. J Cell Biol, 1988. 107(2): p. 545-53 - Janssen, M.E., et al., Three-dimensional structure of vinculin bound to actin filaments. Mol Cell, 2006. 21(2): p. 271-81 We thank Ilona C. for her assistance in the initial stages of the project.
**1124/B282**

**Homozygous Deletion of Pdlim2 Is Associated with Structural Abnormalities of Corneal Epithelia and Increased Levels of Cytoskeletal and Chaperone Proteins.**

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Pdlim2, an E3 ubiquitin ligase, contains an N-terminal PDZ motif and a C-terminal LIM domain. Studies have shown that Pdlim2 is expressed at high levels in corneal epithelium and interacts with α-actinin, filamin A, myosin VI, and MYH9. Furthermore, it is located at actin cytoskeleton and required for epithelial migration. The present study was undertaken to investigate Pdlim2 function in the cornea epithelium. By light and electron microscopy, about 70% of Pdlim2 knockout (KO) mice showed corneal abnormalities, including irregular thickening of the epithelium, calcium deposits along basement membrane, interruptions in the basement membrane, and neovascularization at the stromal/epithelial interface. In addition, KO mice had frequent corneal erosions. To explore possible functions of Pdlim2 in the cornea we compared the corneal protein expression profiles of wild type and KO mice by 2D differential fluorescence gel electrophoresis and analyzed the results using a 2D quantification software. This technique identified 39 spots with more than 2 fold differences. Sequencing and functional annotation of 13 spots identified 43 proteins, with significant clustering in the categories of cytoskeletal proteins and chaperones. Immunoblotting confirmed a 3 to 5 fold increase of certain proteins from both categories in KO mice, including β-actin, destrin, β-tubulin, stathmin 1, tubulin specific chaperone B, αA-crystallin, and αB-crystallin. Examined by Q-PCR, the mRNA level of all these proteins was unchanged, suggesting post-transcriptional regulation. A GFP-Pdlim2 fusion protein co-immunoprecipitated with α-actinin and actin in both transfected corneal epithelial cells and corneal epithelial extracts. GFP fusions with isolated PDZ-, Middle-, and LIM- domains of Pdlim2 will identify specific regions responsible for these and other interactions. TIRF (total internal reflectance fluorescence) microscopy of transfected cells revealed that GFP-Pdlim2 localized to sites of cell-matrix adhesion and actin attachment. Together these findings suggest a role for Pdlim2 as cytoskeletal associated adaptor protein involved in maintaining the normal structure, morphology, and integrity of the corneal epithelium.

**1125/B283**

**Exploring the Functions of FHOD Subfamily Formins Using Caenorhabditis elegans.**

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The Formin HOmology Domain (FHOD) subfamily of formins is highly conserved within the animal kingdom, but the physiological function of this group of actin nucleating proteins is unknown. We are using the roundworm Caenorhabditis elegans as a model system to probe the In Vivo functions of its sole FHOD homolog, FHOD-1. Through a combination of microscopy and mutant studies, we have found that the worm FHOD is enriched in all the muscles of the animal, where it associates with subsets of actin filament bundles, and worms with defects in FHOD exhibit partial defects in the function and morphology of these muscles. Together, these results suggest this conserved formin subtype may be specialized to function in organizing actin in muscle cells.

**1126/B284**

**Z Disc Pathology in Cypher-Null Mice: A 3D Electron Microscopic Study.**

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The Z-disc is a specialized region in vertebrate skeletal muscle where the thick and thin filaments interact with actin and myosin filaments. In this study, we used 3D electron microscopic analysis to investigate the pathology in the Z-disc of Cypher-Null mice, a model for the human disease nemaline myopathy. Our results revealed novel structural defects in the Z-disc, including increased density and size of Z-disc cisternae, and disorganization of the actin and myosin filaments. These findings suggest that Cypher may play a role in maintaining the normal structure and function of the Z-disc in skeletal muscle.
The three-dimensional ultrastructure of the Z disc was studied in the sarcomere of striated muscle of Cypher-null mice. These mice exhibited dilated cardiomyopathy and skeletal muscle failure. Three-dimensional reconstruction of the Z disc was performed on 1 µm thick sections of the diaphragm and on heart tissues using electron tomography in embryonic and new-born Cypher-null mice. Tissue was prepared using conventional heavy metal staining and also using selective staining of the Z disc with ethanolic phosphotungstic acid (EPTA). Both the three-dimensional reconstructions and the selectively stained thick sections of the Z disc demonstrated a discontinuous appearance. The severe fragmentation of the sarcomeric Z disc of a newborn heart and diaphragm of Cypher-null mice had not been documented using previous 2D techniques. Our analysis strongly supports the idea that Cypher plays an important role as a linker-strut in the Z disc of striated muscles. Part of this study was conducted using the Telescience Portal, a tool for enhancing remote collaborations involving large 3D datasets.

1127/B285

Obscurin Determines the Sarcoplasmic Reticulum Structure.

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The giant protein obscurin is thought to link the myofilaments of cross-striated muscle cells with the sarcoplasmic reticulum (SR). The N-terminus of obscurin interacts with the sarcomeric proteins titin and myomesin, whereas the C-terminus mediates interactions with ankyrin proteins. Our objective was to investigate the importance of obscurin for SR architecture and organization by means of creating an obscurin knockout mouse model. Lack of obscurin in cross-striated muscles leads to changes in SR architecture and disruption of small ankyrin-1.5 (sAnk1.5) expression and localization. Reduced expression of sAnk1.5 is due to nedd8- and ubiquitin-dependent protein degradation. Changes in SR architecture in obscurin knockout mice are also associated with alterations in several SR or SR-associated proteins, such as ankyrin-2 and betaspectrin. Obscurin knockout mice display centralized nuclei in skeletal muscles as a sign of mild myopathy, but have normal sarcomeric structure and preserved muscle function.

1128/B286

Troponin I Is Essential for Contractile Regulation of Non-Striated Muscle in the C. elegans Somatic Gonad.

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Troponin is a complex of troponin T, troponin C, and troponin I, which regulates calcium-dependent muscle contraction. Troponin is generally a major actin-linked regulator of contraction of striated muscles, while troponin is not expressed in most of smooth or non-striated muscle. However, troponin is expressed in some types of invertebrate smooth muscle and mammalian vascular smooth muscle, but its physiological function in smooth/non-striated muscle is not clearly understood. The somatic gonad of the nematode Caenorhabditis elegans has non-striated muscle that provides contractile forces during ovulation. Previous studies identified trophin T and trophin C that are essential for contraction of the gonadal non-striated muscle, but trophin I has not been identified. In this study, we demonstrated expression and localization of trophin I in the gonadal muscle by immunofluorescence microscopy and identified two trophin I genes that are required for the function of the gonadal muscle. We found that antibody against Ascaris trophin I stained non-striated filamentous structures in the somatic gonad. These filaments co-localized with actin filaments, indicating that trophin I is a component of the thin filaments. The unc-27 gene encodes a trophin I that plays a major role in striated body wall muscle. In an unc-27 mutant, immunoreactivity of trophin I in the gonad remained intact, suggesting that a trophin I(s) other than unc-27 is expressed. C. elegans has four trophin I genes, tni-1, unc-27 (tni-2), tni-3, and tni-4. We focused on tni-1, unc-27, and tni-3 because tni-4 is specifically expressed in the pharynx. Either an unc-27 mutation or RNA interference (RNAI) of tni-1 or tni-3 in wild-type
background did not cause defects in ovulation. However, RNAi of *tni-1*, but not *tni-3*, in the *unc-27* mutant background markedly diminished immunofluorescence staining of the gonadal muscle by anti-troponin I antibody and caused abnormal reproductive phenotypes. These results strongly suggest that the two troponin I genes, *tni-1* and *unc-27*, have redundant functions for regulating contraction of the gonadal non-striated muscle.

1129/B287
**Molecular Architecture of Synaptic Actin Cytoskeleton in Hippocampal Neurons.**
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Excitatory synapses in the brain play key roles in learning and memory. The formation and functions of post-synaptic mushroom-shaped structures, dendritic spines, and possibly of pre-synaptic terminals, rely on actin cytoskeleton remodeling. However, the cytoskeletal architecture of synapses remains unknown hindering the understanding of synapse morphogenesis. Using platinum replica electron microscopy, we characterized the cytoskeletal organization and molecular composition of dendritic spines, their precursors, dendritic filopodia, and pre-synaptic boutons. We found that bundled actin filaments were rare in dendritic protrusions. Instead, a branched actin filament network containing Arp2/3 complex and capping protein, was a dominant feature of spine heads and presynaptic boutons. Surprisingly, the spine necks and bases, as well as dendritic filopodia, also contained a network, rather than a bundle, of branched and linear actin filaments that was immunopositive for Arp2/3 complex, capping protein, and Myosin II, but not fascin. Thus, a tight actin filament bundle is not necessary for structural support of elongated filopodia-like protrusions. We propose that the network-like organization of the cytoskeleton of dendritic spines and filopodia enhances their plasticity during synapse formation and tuning. Dynamically, dendritic filopodia emerged from densities in the dendritic shaft, which by electron microscopy contained branched actin network associated with dendritic microtubules. We propose that dendritic spine morphogenesis begins from an actin patch elongating into a dendritic filopodium, which tip subsequently expands via Arp2/3 complex-dependent nucleation and which length is modulated by Myosin II-dependent contractility. Supported by NIH grant GM 070898.

1130/B288
**Neuronal Localization of Dystonin-A2 Isoform: A Possible Role for Maintaining Endoplasmic Reticulum Integrity?**
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Dystonia musculorum (dt) is an inherited neurodegenerative disease in mice that leads to sensory ataxia. While the underlying mechanisms leading to sensory neuron degeneration in dt mice are currently unclear, it has been postulated that mutations within dystonin-Bpap1 has a role in the disease pathology. Dystonin Bpap1 is a member of the plakin family of proteins that is involved in cross-linking cytoskeletal elements and attaching them to cell junctions. Dystonin Bpap1 is alternatively spliced to produce several neuronal isoforms, including dystonin-a1, dystonin-a2 and dystonin-a3. The neurological phenotype displayed by dt mice results from the absence or aberrant functioning of dystonin-a isoforms in the developing nervous system. Recent studies using dystonin-a2 fusion constructs revealed that this isoform is involved in nuclear envelope (NE) structuring, nuclear tethering, and organization of membranous structures surrounding the nucleus (i.e., endoplasmic reticulum). To determine the endogenous localization of dystonin-a2, an antibody targeting the N-terminus was employed. To validate antibody specificity we transfected Cos-1 cells with dystonin-a2 fusion constructs and observed co-localization. Using neuronal F11 and PC12 cell lines in both the undifferentiated and differentiated states, we detected perinuclear and perikaryal staining with the a2-antibody. In addition, we detected a co-localization between the a2-antibody staining and the endoplasmic reticulum (ER) marker protein disulfide isomerase (PDI). Primary dorsal root ganglion (DRG) cells of P5 wild type mice also exhibited a perikaryal staining pattern; however, some P5 (pre-phenotype stage) dt DRGs manifested aberrant PDI localization and showed an increase in PDI fluorescence. The
increased PDI fluorescence may indicate an ER stress response and this may augment neurodegeneration. Our preliminary results demonstrate that dystonin-a2 localizes throughout the cytoplasm, likely associating with the ER and NE, suggesting the absence of dystonin may result in ER stress triggering neurodegeneration. (Supported by a grant from the CIHR).

1131/B289
Distribution of Dystrophin- and Utrophin-Associated Protein Complexes (DAPC/UAPC) during Activation of Human Neutrophils.
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Introduction and Background: Human neutrophils are key effector cells of the innate immune system. They migrate rapidly to sites of infection, become activated, and initiate a cascade of defense mechanisms against microbial infection in which locomotor and adhesive functions involve a functional actin network that serves as a force-generating mechanism. Chemotactic activation of neutrophils is accompanied by a dramatic reorganization of actin filaments, and actin-binding proteins stabilize the actin network at the lamellipodia. Dystrophins, utrophins, and their associated proteins are involved in structural and signaling roles in non-muscle tissues; however, description of these proteins in neutrophils remained unexplored. Objective: In the present study, we characterize the pattern expression and the subcellular distribution of dystrophin and utrophin gene products and dystrophin-associated proteins (β-dystroglycan, α-syntrophin, α-dystrobrevins) in relation to actin filaments in resting and activated human neutrophils. Methods: Immunoblot, confocal microscopy analysis and immunoprecipitation assays. Results: Our results demonstrated the presence of two dystrophin-associated protein complexes the Dp71d/Dp71Δ110m~DAPC and the Up400~DAPC in lamellipodia and podosome in activated neutrophils. Conclusions: The presence and redistribution of the two dystrophin-associated protein complexes corresponding to dystrophins and utrophin in activated neutrophils suggest their dynamic participation in the chemotaxis process.

1132/B290
Signaling and Structural Organization of Nonmuscle Myosin in Mesenchymal Stem Cells.
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Mesenchymal Stem Cells (MSCs) appear exquisitely sensitive to the mechanical properties of their microenvironment, with lineage specification even directed by matrix elasticity, but initiating mechanisms remain largely obscure other than a role for contractility. One of the earliest changes in MSCs on substrates of different elasticity is ordering of stress fibers, and disruption of this cytoskeletal ordering by inhibition of non-muscle Myosin II with blebbistatin occurs in minutes and, over days, blebbistatin also blocks MSC differentiation. Both cytoskeleton and adhesions are well known to be regulated by tyrosine kinase signaling, and direct signaling to myosin heavy chains with rapid effects on cytoskeletal ordering define a potential but ill-defined pathway in mechano-sensing. We have addressed the role of tyrosine phosphorylation in regulating the major non-muscle Myosin II in MSCs, Ila (Myo2a), and we relate this signaling to myosin localization dynamics by combining fluorescence microscopy with Atomic Force Microscopy (AFM) imaging. After growing MSCs on collagen I-coated substrates for brief periods (1, 4, 24hrs) and extracting the membrane and cytosolic proteins, we show that the intensity of Myo2a fluorescence correlates closely with the height of cytoskeletal filaments in AFM images. Importantly, the density and organization of the filaments increase over time in parallel with phospho-tyrosine in MSC lysates. Two sites previously reported to be phosphorylated in Myo2a...
were mutated (Y1805F and Y277F), and the mutants show poor incorporation into stress fibers in comparison to WT Myo2a-GFP. Their fluorescence intensity also correlates poorly with the height of cytoskeletal filaments in AFM images. The results implicate tyrosine kinases in direct regulation of Myo2a filament assembly and critical control of cytoskeletal organization in MSCs.

1133/B291
Spatial Control of Polarized Cell Growth in Fission Yeast S. Pombe.
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The spatial regulation of polarized cell growth is essential for normal cell function and differentiation. Here, we present a detailed analysis of a data set of cell growth measurements in Schizosaccharomyces pombe, which compare the separate growth patterns of the old and new ends. We find that old and new cell tips display different growth rates that positively correlate with the local distribution of active GTP-bound Cdc42. Furthermore, we find that tip growth is modulated by the presence of another growing tip, indicating competition between different growth zones. Intensity quantifications of active GTP-bound Cdc42 at the cell cortex are also consistent with competition between growing cell tips.

1134/B292
Shaping Yourself with Autophagy: A Novel Role for Autophagy in Cell Shape Regulation.
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The cellular “self-eating” process of autophagy is critical to cell homeostasis and has profound consequences on cellular physiology during stress and disease. We describe a novel function for autophagy in regulated cell shape changes and cytoskeletal remodeling. Drosophila blood cells, called hemocytes, akin to their mammalian macrophage counterparts, require regulated cell shape changes to carry out key roles in innate immunity, wound healing and developmental remodeling. In response to various cues, hemocytes and macrophages undergo a characteristic cell spreading, involving cell flattening and the extension of dynamic protrusions. We demonstrate that blocking autophagy, either through genetic disruption of autophagosome formation or by the use of chemical inhibitors of autophagy, abolishes spreading in Drosophila hemocytes. The loss of spreading in autophagy-blocked hemocytes is cell autonomous and not a consequence of increased cell death. Temporal studies with the drug 3-Methyladenine (3-MA, a potent inhibitor of autophagy) suggests that autophagy is required both for the initiation as well as the continuous maintenance of the characteristic morphology of spread hemocytes. While autophagy-blocked hemocytes lack extended protrusions, they still exhibit a dynamic cortex. Signaling through the Rho, Rac and Cdc42 GTPase pathways regulate distinct cytoskeletal architectures. In epistasis studies with activated GTPase forms, we found that autophagy was required for the Rho-driven formation of F-actin based protrusions in hemocytes. We also found that mouse macrophage cell lines (RAW and J774) require autophagy to undergo a characteristic cell elongation in response to specific stimuli. Thus, there seems to be a conserved role for autophagy in regulating macrophage cell spreading. We are now elucidating the molecular mechanisms that underlie the nexus between autophagy and the cellular remodeling required for blood cell spreading, furthering our understanding of two important processes that profoundly affect cellular physiology and functions in health and disease.

1135/B293
Effect of Hyperglycemia on Cytoskeleton of Osteoblasts.
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During the development of diabetes mellitus are present several complications by hyperglycemia, such as damage bone, therefore, osteopenia and osteoporosis are associated with this disease.
These alterations are based in abnormalities on bone metabolism, fundamentally, by misbalancing in bone formation and resorption, and so, decrement in bone mineral density. Furthermore, other cells exposed to hyperglycemia have changes in structure, proliferation, differentiation and sometimes, present apoptosis. Particularly, on osteoblasts have been studied some alterations by hyperglycemia, however, unknown the influence on cytoskeleton and response under the treatment with insulin. These aspects were studied in this work. We used osteoblastic cells (MG-63, ATCC CRL-1427) which were incubated in Dubelcco’s Modified Eagle’s Medium supplemented with 10% Fetal Bovine Serum containing either a normal (5 mM) or high glucose concentration (15 and 25 mM) with or without insulin (0.6 mM) to 48 and 72 h.

The morphologic changes, nucleus and actin filaments were analyzed by staining with DAPI and phalloidin-FITC. The results show that osteoblasts cells exposed to insulin and 5 mM glucose present short filaments accumulated in periphery cell, represented by fluorescence spots, in contrast stress fibers were observed in osteoblasts exposed to 15 and 25 mM glucose, concentrated in the middle cell zone. Some cells changed from typical osteoblastic morphology to round off, changes phenotypically confirmed. These observations were present every exposition time. No nuclear alterations were detected in every experimental conditions. These data suggest that the insulin could be a fine agent to avoid osteoblastic damage. Additional studies will address the signaling pathways of cytoskeletal changes, that will permit to have a better understanding about diabetes and bone.

**Cell Migration II (1136 – 1164)**

**1136/B294**

**Regulation of Cell Migration by Caldesmon Phosphorylation May Control Tumor Metastasis.**

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It has been shown previously that phosphorylation of the actin-binding protein, caldesmon (CaD), by cdc2 kinase was sufficient to increase the migration activity in HeLa cells (Manes et al. J Cell Biol 2003;161:817-26). Recently we have found by mutagenesis that phosphorylation of CaD at the ERK or PAK sites is also necessary for maintaining enhanced cell migration in breast cancer cells. Taken together, these observations strongly suggest that ERK/PAK signaling via the downstream effector CaD plays a key role in controlling the cell migration activity. We further noticed that not only the human metastatic breast cancer cell line MDA-MB231 contains a significantly higher amount of CaD compared to the non-metastatic cell line MCF-7, but the ERK-mediated CaD phosphorylation thereof is also more extensive than in the normal mammary epithelial cells. This is consistent with the fact that MDA-MB231 cells carry the K-ras mutation, and as a consequence, these cells exhibit a higher metastatic activity. Interestingly, several other aggressive tumor cell lines, such as HS578T (human breast cancer) and SNB-19 (human glioblastoma) were also found to express high levels of CaD as well as to have constitutively activated ERK and/or PAK pathways. The combination of elevated CaD content and kinase activity thus appear to be critical factors for the highly invasive and migratory behaviors of metastatic tumor cells. In another study we have found that CaD, when phosphorylated at the ERK sites, moves to the cell leading edge, where it may stabilize nascent actin filaments and promote actin dynamics. Such properties could have important bearings to the relationship between CaD and tumor metastasis. It would be interesting to test the CaD expression and phosphorylation level in other types of malignant tumor cells. Our data suggest CaD could serve as a therapeutic target of metastatic cancers.

**1137/B295**

**Shifting the Mechanism of Cell Migration Participates in Pulmonary Metastasis of Osteosarcoma.**

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Objective: Outcomes for patients of osteosarcoma with pulmonary metastasis still remain poor. Thus, the elucidation and suppression of the mechanism inducing pulmonary metastasis of osteosarcoma is strongly needed. Methods: We previously established highly pulmonary metastatic cell line, LM8 from Dunn mouse osteosarcoma through In Vivo selection. LM8 cells revealed higher MMP-2 secretion, VEGF production, and invasion property compared to Dunn cells. In the current study, we further investigated the biological features of highly metastatic osteosarcoma using this syngeneic mouse model. Results: LM8 cells showed higher motility as to horizontal and vertical migration, and also higher Cdc42 activity and autophosphorylation level of focal adhesion kinase compared to Dunn cells. LM8 cells acquired striking filopodia and localized integrin β1 on the cell periphery, but showed reduced actin stress fiber and focal adhesion formation. A specific Rho kinase inhibitor, Y-27632 (10 μM) increased LM8 migration with increased Cdc42 activity, while it reduced Dunn migration in Boyden chamber migration assay. By contrast, the knockdown of Cdc42 using siRNA silencing reduced LM8 migration, and the transfection of constitutively active V12Cdc42 mutant increased Dunn migration. We next screened clinically approved anticancer drugs and found that irinotecan suppressed the migration and Cdc42 activity of LM8 cells, although these effects of irinotecan were marginal in Dunn cells. Daily oral administration of irinotecan significantly reduced the rate and size of pulmonary metastasis of LM8 in syngeneic C3H mice. Conclusions: The up-regulated Cdc42, maintained by signaling network linking extracellular matrix with Cdc42, and resistance to Y-27632 in migration suggest that shifting the mechanism of cell migration induces the high motility and metastatic ability of LM8. Irinotecan returns this change of motility mode and could be a candidate drug for preventing pulmonary metastasis of osteosarcoma.

1138/B296
Specific Cortactin Tyrosine Phosphorylation Sites Regulate Actin Polymerization and Matrix Degradation during Invadopodium Maturation.
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Invadopodia are specialized membrane protrusive structures found unique to metastatic carcinoma cells that have the ability to degrade the underlying extracellular matrix. Assembly and maturation of invapodia is a multi-step process that requires cortactin phosphorylation leading to cofilin activity and Arp2/3 complex-dependent actin polymerization. The precise role of the individual phosphorylation sites of cortactin during invadopodium maturation is not understood. We have analyzed the contribution of individual tyrosine phosphorylation residues 421, 466 and 482 to invadopodium precursor formation, free barbed end formation, and matrix degradation by invadopodia. We show that cortactin tyrosine phosphorylation is not necessary for invadopodium precursor formation. Importantly, phosphorylation of tyrosine residues 421 and 466, but not 482 are important for free barbed end formation in invadopodia downstream of EGF stimulation. Similarly, phosphorylation of the same tyrosine residues, 421 or 466, is required for efficient matrix degradation. In particular, we show that the binding of Nck1 to cortactin is reduced in the 421 and 466 mutants at invadopodia. Similarly we show that cofilin’s binding interaction with cortactin and local activation is regulated by phosphorylation of these residues. These findings identify the 421Y and 466Y phosphorylation sites as the key regulators of cortactin mediated actin polymerization and matrix degradation.

1139/B297
The Protein Convertase Furin in Human Trophoblast: Possible Role in Promoting Trophoblast Cell Migration and Invasion.
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Furin, a proprotein convertase (PC), is ubiquitously expressed and implicated in many physiological and pathological processes. This study is aimed to identify the role of furin in human trophoblast invasion and migration. Furin was found to be highly expressed in placental villi of both rhesus monkeys and human beings during early pregnancy. Specifically, furin was found in trophoblast column and trophoblast shell, regions which give rise to highly invasive cytotrophoblast cells to invade the maternal decidua during human placentation. To determine whether furin plays any role in trophoblast invasion and migration, we employed human extravillous HTR8/SVneo cells in Matrigel invasion and transwell migration assays. Knocking-down furin expression by siRNA significantly inhibited invasion and migration of HTR8/SVneo cells (P<0.01), with corresponding decrease of matrix metalloproteinase-9 (MMP-9) activities. In contrast, over-expression of furin markedly increased cell invasion and migration (P<0.01), accompanied by significant increase of MMP-9 activities. Furthermore, furin siRNA significantly increased the levels of both tissue inhibitors of MMPs (TIMP)-1 and -2. Our results suggest that furin may play an important role in the invasion and migration of human trophoblast cells during early pregnancy.

1140/B298
Actomyosin Bundling and Adhesive Signaling Regulate Initial Front-Back Polarization in Migrating Cells through Myosin IIIB.
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Migrating cells polarize by forming a protrusion in the direction of migration, and a rear region that retracts. Here we present evidence that non-muscle Myosin II (NM II) generates front-back polarity and controls microtubule and Golgi polarization. Localized NM II activation induces the formation of a "primordial" actomyosin bundle that locally inhibits protrusion. This structure defines the polarity of the cell by creating the rear. The stability of this actomyosin bundle is NM II isoform-specific. NM II-A-generated bundles consecutively assemble and disassemble rapidly, locally blocking and allowing protrusion. In contrast, NM II-B or II-C forms stable bundles. Isoform specificity appears to rely, in part, on the differential phosphorylation of the tail regions, which regulates actomyosin filament formation. Stability also depends on the nature of NM II-B activation by phosphorylation of the regulatory light chain (RLC). Mono-phosphomimetic RLC increases actin bundling; however, these bundles can disassemble and allow protrusion. In contrast, diphosphomimetic RLC acting on NM II-B induces highly stable bundles that define the rear of the cell. Similar effects are also observed in adhesions; monophosphomimetic RLC increases adhesion maturation throughout the cell, whereas diphosphomimetic RLC induces large, stable adhesions at the rear and sides. Finally, NM II activation by diphosphomimetic RLC locally shuts down tyrosine phosphorylation of paxillin (Y31, Y118) and FAK (Y397). These effects of diphosphomimetic RLC are similar to those of a contraction-inhibited mutant of NM II-A (N93K) that is locked in an actin-bound state. Constitutively active Rac inhibits formation of the primordial actomyosin bundle and front-back polarity and relieves MII-mediated inhibition of tyrosine phosphorylation in adhesions. We propose a model in which activation of NM II-B (or II-C) generates an initial actomyosin bundle that eventually becomes the rear as the cell spreads away from it. Protrusion from this area is inhibited by the local suppression of adhesive signals that activate Rac.

1141/B299
Cdk5 Regulates Rho-Dependent Cytoskeletal Contraction and Epithelial Cell Migration by Suppressing Activities of Src and P190RhoGAP.
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Cell migration is a highly integrated, multi-step process essential for embryogenesis and for growth, renewal, and wound healing throughout life. Cdk5, a Ser/Thr kinase, regulates adhesion and migration in variety of cell types. We recently showed that stress fiber formation and contraction are associated with elevated Cdk5 activity. Here we test whether Cdk5 activity...
regulates myosin phosphorylation essential for these observed effects and investigate possible mechanisms by which Cdk5 regulates cell migration. Inhibiting Cdk5 activity with olomoucine, siRNA, or dominant negative Cdk5 significantly reduced phosho-myosin regulatory light chain (pMRLC) level and cell contraction. This was accompanied by loss of central stress fibers, and reduced pMRLC immunofluorescence. Blocking Cdk5 activity with olomoucine or siRNA reduced Rho-GTP level and Rho kinase (ROCK) activity, indicating that Cdk5 regulates MRLC phosphorylation by controlling Rho-ROCK signaling. To determine whether Cdk5 exerts its effect on cell migration via Rho-ROCK signaling, we assessed the effect of olomoucine on scratch wound closure in the presence or absence of the ROCK inhibitor, Y-27632. Olomoucine and Y-27632 increased cell migration to an equal extent. Addition of both inhibitors together did not show any additional effect, demonstrating that Cdk5-dependent regulation of ROCK activity is sufficient to explain the observed effect of Cdk5 inhibition on migration. Src is a potential substrate of Cdk5, which regulates Rho activity in migrating cells by phosphorylating and activating p190RhoGAP, an upstream inhibitor of Rho. Inhibiting Cdk5 activity increased both Src activation and phosphorylation of its substrate, p190RhoGAP, demonstrating that Cdk5 controls Rho by regulating Src and p190RhoGAP activity. Moreover, the Src inhibitor PP1 completely reversed the effect of Cdk5 inhibition on Rho activation, cytoskeletal organization, and Rho-dependent myosin contraction. These findings demonstrate that Cdk5 exerts its effect on epithelial cell migration and Rho-dependent myosin contraction by regulating activities of Src and p190RhoGAP and reveal that Cdk5 is a key regulator of myosin phosphorylation, cytoskeletal contraction and cell migration.

1142/B300
Function of Nonmuscle Myosin II Isoforms in LPA1-Induced Migration and Invasion.
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The enhanced migration found in tumor cells is often caused by external stimuli and the sequential participation of cytoskeleton-related signaling molecules. However, until now, the molecular connection between the lysophosphatidic acid (LPA) receptor and cell migration has not been analyzed for the LPA-induced mouse breast cancer line, 4T1. In this study, we demonstrate that LPA induces migration by activating the LPA1 receptor which promotes phosphorylation of the 20kDa nonmuscle Myosin II light chain through activation of Rho kinase (ROCK). We show that LPA-induced migration is insensitive to pertussis toxin but does require the LPA1 receptor as determined by siRNA and receptor antagonists. LPA activates ROCK as a downstream effector of RhoA and also increases GTP-bound RhoA activity, concomitant with the enhanced membrane recruitment of RhoA. LPA-induced migration and invasion are attenuated by specific inhibitors including the C3 cell-permeable transferase and the ROCK inhibitor, Y-27632. We demonstrate that nonmuscle Myosin II (NM II) plays an important role in LPA-induced migration and invasion by inhibiting its cellular function with blebbistatin and shRNA lentivirus directed against the NM II-A or NM II-B heavy chain, to stably silence NM II-A and NM II-B expression. Surprisingly, and in contrast to previous findings using MCF 10A (Even-Ram et al. Nat. Cell Biol. 2007, 9:299-309) inhibition or loss of either NM II-A or NM II-B heavy chain, to stably silence NM II-A and NM II-B expression. Presently we are investigating the mechanism underlying these apparent differences in cell behavior.

1143/B301
Novel Form of Epithelial Cell Migration Depends on Myosin IIA to Establish Cell Polarity and Retrograde Flow.
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Epithelial cell migration is an essential part of embryogenesis and tissue regeneration, yet their migration is least understood. Our three-dimensional motility analysis revealed that, unlike any other migrating cells, epithelial cells migrate with a constant, polarized cell shape with the nucleus leading the cell front. Surprisingly, migrating epithelial cells contracted the anterior and posterior extracellular matrix simultaneously and symmetrically. The anterior matrix deformation was due to retrograde flow generated by the cell cortex, and the posterior matrix deformation was due to the strong adhesion between migrating cells and the matrix. Despite the similar sub-cellular localization of Myosin II isoforms, cell polarization and force generation depended on Myosin IIA but not on IIB. Interestingly, on a two-dimensional substrate, cells with reduced level of Myosin IIA migrated faster than wildtype cells, but in a three-dimensional gel, these cells did not polarize and were immobile. Our three-dimensional migration assay demonstrates that Myosin IIA, and not IIB, is required for this novel form of epithelial cell migration.

1144/B302
Regulation and Dynamics of Myosin-II Activation during Epidermal Wound Responses.
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The molecular signals and underlying mechanisms that stimulate Myosin II activity during epidermal wound healing have not been elucidated. We have uncovered signaling pathways involved in the activation of Myosin II in migrating primary human keratinocytes in response to scratch wounding in vitro. We report here that Myosin II activation and assembly in wounded keratinocytes is biphasic. Post-wounding, a rapid phosphorylation of Myosin II regulatory light chain (RLC) and resultant filament assembly occurs far in advance of the later cell migration events that are triggered by the scratch wounding. During this acute-phase activation of Myosin II assembly, pharmacological approaches reveal p38-MAP kinase and cytosolic calcium as having critical roles in the phosphorylation driving filament assembly. Although p38-MAPK has known roles in keratinocyte migration, and known roles in leading-edge focal complex dynamics, to our knowledge this is the first report of p38-MAPK acting as an upstream activator of Myosin II phosphorylation and assembly.

1145/B303
Membrane Phosphoinositide Availability, Regulated by Profilin-1, Determines Breast Cancer Cell Motility Secondary to Binding of Lamellipodin.
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Objective: Expression of profilin-1 (Pfn1) is significantly downregulated in various invasive adenocarcinoma including breast cancer cell. The objective of this study is to elucidate molecular mechanisms of how loss of expression of a pro-migratory molecule like Pfn1 actually enhances breast cancer cell migration. Results: We show that loss of Pfn1 expression increases motility of MDA-MB-231 (MDA-231) breast cancer cells by enhancing targeting of VASP to the leading edge. We show that VASP targeting to the leading edge is mediated through the action of Lpd and Pfn1 negatively regulates membrane targeting of Lpd. Analyzing motility of various ligand-binding deficient mutants (actin, polyproline, phosphoinositide) of Pfn1 in a knockdown-knockin setting, we further demonstrate that hypermotile response and Lpd/VASP distribution in Pfn1-deficient cells are best phenocopied by the phosphoinositide-binding deficient mutant of Pfn1. Motility of cells bearing phosphoinositide-binding deficient mutant of Pfn1 is extremely sensitive to both Lpd depletion and VASP inhibition. Finally, using multiple experimental strategies including PI3-kinase inhibition, PTEN overexpression and expression of PH-AKT, we show that Lpd-rich lamellipodial phenotype of Pfn1-depleted cells is critically dependent on the availability of D3-phosphoinositides. Conclusions: These results lead to a novel paradigm that Pfn1, although generally conceived as a pro-migratory molecule, can suppress mammary carcinoma cell motility by regulating membrane availability of Lpd through competition and this involves Pfn1’s
phosphoinositide interaction. This is in contrast to conventionally thought Pfn1’s role in cell motility mainly through its actin and polyproline interactions (Funded by CA 108607 to PR).

1146/B304
Cell Polarity-Related Septin Proteins, Sept14 and Sept4, Regulate Neural Cell Positioning and Shape during Brain Development.
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Correct neuronal generation, migration and positioning during cortical development are essential for proper brain functions. Septins are cytoskeleton-related GTP/GDP-binding proteins thought to be involved in cell cycle, cell polarity and signal transduction. Some septins are expressed in specific manners in developing neuronal tissues, raising the possibility that septins are functionally involved in neuronal development. We focused on a septin family member, Sept14, and examined the protein expression profile during mouse brain developmental process. In western blotting, Sept14 came to be visualized at E16.5, and the expression was then increased during the development. In immunohistochemical analyses, Sept14 was specifically expressed in cells in the cortical plate of developing brain. We next examined the effects of Sept14 on the positioning of cortical neurons. Knockdown (KD) of Sept14 in neuroepithelial cells by in utero electroporation resulted in defective cell positioning and cell shape with abnormal polarity. It, however, seemed that the abnormally positioned cells were differentiated normally as they expressed a neuronal differentiation marker, NeuN. To understand the molecular basis of the Sept14 function in the neuronal positioning and morphology during corticogenesis, we screened interacting partners for Sept14 and identified another septin molecule, Sept4. Interestingly, Sept4-KD induced phenotypes very similar to those observed in Sept14-KD; abnormal cortical neuron positioning and cell shape during brain development. Moreover, phenotypes of Sept14-KD were rescued by RNAi-resistant Sept14 (RNAi-R-Sept14) but not by a RNAi-R-Sept4 mutant lacking the C-terminal coiled-coil region essential for the interaction with Sept4. These results suggest that Sept14 and Sept4, in a coordinated manner, play important roles in the brain development through neuronal cell polarity formation and maintenance.

1147/B305
Bacillus Anthracis Edema Toxin Impairs Neutrophil Actin-Based Motility by the Phosphorylation of VASP.
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Inhalation anthrax can lead to sepsis and death within a matter of days. The fulminate nature of illness reveals normal to minimal elevation of peripheral polymorphonuclear (PMNs) counts at the time of hospital admissions indicating a profound impairment of the innate immune response. Neutrophils are the primary cell component of the innate immune response and are the earliest responders to the invasion of bacterial pathogens. We have previously shown that anthrax edema toxin (ET) impairs neutrophil motility and the actin based motility of the intracellular pathogen Listeria monocytogenes. Edema toxin’s inhibition of actin assembly is mediated by the ability of this toxin to phosphorylate the Vasodilator-stimulated phosphoprotein (VASP) at serine 157. VASP is a cAMP dependent protein kinase a (PKA) substrate, which links cellular signaling to cytoskeletal organization and cellular movement. We propose that the increase in cAMP levels caused by ET, an adenylate cyclase toxin, activates PKA allowing for the downstream phosphorylation of VASP at serine157 leading to the decrease in motility. Stimulation of various cell lines with ET shows a significant increase in phosphorylated VASP which can be mimicked by the combination of forskolin and IBMX (adenylate cyclase activating compound and a non-specific cAMP phosphodiesterase inhibitor). Delivery of pseudo-phosphorylated VASP at S157 inhibits motility mimicking the effects seen with ET, while pseudo-unphosphorylated VASP at S157 does not decrease motility. The effects of ET on VASP phosphorylation and actin assembly emphasize the importance of the cAMP signaling pathway and VASP for actin based motility.
These findings demonstrate that anthrax ET can inhibit actin-based assembly, and these effects may help to explain the meager neutrophil response that accompanies the early stages of systemic anthrax.

1148/B306
Allosteric Control of Alpha-Catenin in Cell Adhesion and Migration.
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The Adherense junctions (AJs) are essential for cell-cell adhesion and play an important roles in cell migration and epithelial-mesenchymal transition during embryogenesis, wound healing and cancer. The AJs comprise members of the cadherin family, transmembrane cell adhesion proteins that bind through their cytoplasmic domain to p120 and β-catenin; and β-catenin in turn binds to α-catenin (α-cat). The textbook model of the AJ depicts a stable complex in which the AJ is bound to the actin cytoskeleton, with the α-cat providing the molecular link between the actin cytoskeleton and the AJ complex. A direct test of this model showed, however, that α-cat cannot form a quaternary complex with cadherin, beta-catenin and actin. Instead α-cat behaves as an allosteric protein in which monomeric α-cat is able to form a ternary complex with cadherin/β-catenin, but not actin, while dimeric α-cat binds to and bundles actin filaments. α-cat is in dynamic equilibrium between monomer and dimer pools and it has been proposed that a local increase in α-cat concentration during clustering of the cadherin/catenin complex could regulate local formation of α-cat dimers in the cytoplasm. These results point towards a dynamic, rather than static role of α-cat in locally regulating actin and membrane dynamics at sites of cell-cell adhesion. However, the new α-cat’s properties were shown by In Vitro studies. We chose the zebrafish gastrulation to verify the new α-cat properties in vivo. We are using, together with the morpholino approach, a number of constructs that are able to mislocalized the α-cat interfering with his allosteric regulation. These constructs are able to direct α-cat to the mitochondria or to the plasma membrane (in a AJs independent manner). The rational of this approach is to perturb dynamically the α-cat concentration of the different pools. Our preliminary results shows that the morpholino caused delayed or arrested in epiboly and the embryos do not complete somitogenensis. Inducing mitochondria mislocalization of α-cat does not delay epiboly. However, the injected embryos display morphological defects.

1149/B307
Role of Short and Long Tropomyosin Isoforms as Actin Decorators at the Leading Edge of Migrating PtK1 Cells.
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The cortical network formed by actin filaments at the leading edge of cells is part of a complex polarized machinery involved in cell protrusion. It comprises two F-actin modules: the lamellipodium and the lamella, as defined by their different actin dynamics and by the presence of at least two other key proteins, cofillin and Arp2/3. The relationship between these two networks is still unclear. The aim of this study is to investigate the role of multiple tropomyosin (TM) isoforms in the lamella and lamellipodia networks. Our working hypothesis predicts that the formation of a dendritic lamellipodium network nucleated by Arp2/3, requires a subset of lamella actin filaments to escape the decoration by tropomyosin. Immunoblotting analysis of total cell extracts using a variety of specific anti-tropomyosin antibodies suggests that both long (αTM2 and αTM3) and short (γTM5NM1/NM2 and δTM4) tropomyosin isoforms are present in PtK1 epithelial cells; this result was further supported by mass spectrometry analysis and cloning of the individual tropomyosins from PtK1 total RNA. We prepared cell extracts isolated from cell pseudopodia and analyzed their tropomyosin content by immunoblotting. This analysis suggests that at least one member of the long and short isoforms is present within the first 5-10 μm from the cell edge; this result was further confirmed by mass spectrometry analysis. Moreover, we fluorescently-labeled
one long (αTM2) and two short (γTM5NM1 and δTM4) recombinant rat tropomyosins and analyzed the correlation between the dynamics of tropomyosin and actin in the lamella and lamellipodia networks using Fluorescent Speckle Microscopy. We also correlated the dynamics of long and short tropomyosin speckles within the same regions. The multivariate analysis of tropomyosin isoforms with F-actin in protruding cells supports the idea of transient decoration of lamellipodium filaments by short TM isoforms, and more stable decoration of lamella filaments by long TMs, with δTM4 as the tropomyosin isoform localizing at the very cell edge followed by γTM5NM1. Supported by R01GM71686 (GD), R01GM63257 (SEHD), SNF PBZHA-112712 (AB)

1150/B308
Actin Bundling Is Essential for Directional Cell Migration.
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Directional cell migration is essential for embryonic development and in adult tissue it contributes to wound healing and pathological conditions such as metastases. In this study, we identify an epithelial cell-specific actin-bundling protein that regulates directional cell migration. Villin is an epithelial cell specific actin-binding protein that regulates cell migration both In Vitro and in vivo. Villin is unique among other actin regulatory proteins in that it can nucleate, cap, sever and bundle actin filaments. The specific actin modifying function of villin that is required for cell migration however, remains to be determined. In this study we demonstrate for the first time, that actin bundling by villin is required for filopodial assembly and therefore for directional cell migration. We have previously demonstrated that villin self-associates and that villin self-association is required for actin-bundling by villin. In this study we demonstrate that villin’s ligand-binding property namely its ability to bind phosphatidylinositol 4,5-bisphosphate (PIP2) and F-actin is required for villin self-association, for villin’s ability to assemble filopodia and for directional cell migration. Both In Vitro and in cells, increasing concentrations of PIP2 and F-actin increased villin dimers as well as filopodial assembly. Further, we demonstrate that villin mutants that failed to dimerize and therefore fail to bundle actin failed to assemble filopodia and lacked directional migration. By elucidating the role of villin in filopodial assembly, our studies provide a molecular mechanism for regeneration, maintenance of the epithelium and metastases.

1151/B309
How Cells Crawl: Advance of the Leading Edge Mediated by a Continuous Actin Filament System.
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There is much debate over the nature of the connection between the actin filament networks of the lamellipodium and lamellae at the leading edge of crawling cells. Previous studies have suggested that the fast retrograde actin flow zone of the lamellipodium and the slower retrograde flow of the lamellae are functionally distinct but may overlap spatially and temporally. We investigated whether the lamellipodium and lamellae should be considered as two separate or one continuous actin network(s). Electron microscopy of rotary shadowed PtK1 cells did not reveal a break in the continuity of the actin networks but instead showed a transition between the branched actin of the lamellipodium to the bundled actin arc population that defines the lamellae. Time-lapse imaging of actin-RFP during the cycle of leading edge protrusion and retraction showed the appearance of a newly formed (nascent) actin arc coincident with a condensation of the lamellipodium during each event of edge retraction. To test whether the actin arcs of the lamellae were created from the actin filaments of the lamellipodium, we photo-converted actin-EOS molecules in the lamellipodium during edge protrusion and found the same actin filaments were also incorporated into the nascent actin arc formed after the next edge retraction. Thus, the actin network at the leading edge is continuous. Specifically, the actin network of the lamellipodium creates the lamellae. Nascent actin arc formation is Myosin II-dependent. Myosin
II, which usually localizes in the lamellae, also appears at the leading edge and associates with every nascent actin arc during edge retraction. Finally, each nascent actin arc slows down as it couples to focal adhesions, and this is followed by a leading edge protrusion event. Coupling of nascent actin arcs to focal adhesions, therefore, appears to set the base for lamellipodial protrusion and retraction. New focal adhesions form between old focal adhesions and the leading edge. We report that when new focal adhesions form, nascent actin arcs couple to them and set a new base for the protrusion and retraction cycle. This results in the net advance of the leading edge allowing for directed cell crawling.

1152/B310
B1 Integrin Activity and Turnover Is Involved in Ret Receptor Mediated Cell-Migration.
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The RET receptor tyrosine kinase is an important mediator of cell-growth, differentiation, and migration of neural crest derived cells. RET signaling can be disrupted in the presence of well characterized disease-associated mutations. The phenotypes of these diseases, Multiple Endocrine Neoplasia type 2 and Hirschsprung Disease, both indicate that normal cell-migration is also affected by alterations in RET. Here, we examine the contribution of β1 integrin (ITGB1) to RET-mediated cell migration. Integrins are heterodimeric proteins required for cell migration due to their ability to mechanically attach cells to extracellular matrix (ECM) and to transmit signals through focal adhesion (FA) formation. We used standard cell-adhesion and migration assays, in combination with co-immunoprecipitations and confocal-microscopy, to determine the effects of RET on ITGB1-mediated cellular processes. Initially, we found that the presence of ECM enhances RET-mediated cell-migration, suggesting that integrin family members could play a role in RET-mediated migration. Upon activation of RET, cell-adhesion to ECM and formation of ITGB1-paxillin complexes increased, suggesting that ITGB1 is activated downstream of RET. However, after 1 hour, these effects diminish, even in the presence of sustained RET activation. We postulate that these decreases are associated with focal adhesion turnover and internalization, a property necessary for cell-migration. Consistent with this, we saw that cell-adhesion increased again after 12 hours of RET activation. Also, ITGB1 transcript and protein levels were elevated after 24 hours of RET stimulation, suggesting that sustained RET activation refreshes ITGB1 levels. Our data show that RET activation elicits both an early and late response of ITGB1 and, further, that the effects of RET on ITGB1 are two-fold. First, RET is able to mediate ITGB1 activity, leading to increased adhesion and ITGB1-paxillin binding, and secondly, RET is able to modulate expression levels of ITGB1. These results, in combination with future studies utilizing disease-associated RET, will clarify the role of cell-migration in RET-associated diseases.

1153/B311
βPix Null MEFs Show Defects in Dorsal Ruffle Formation and Directional Migration.
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βPix(Pak interacting exchange factor) is known as a guanine nucleotide exchange factor(GEF) for Rho family small GTPases, Rac1 and Cdc42. Overexpression and knockdown experiments suggest that βPix is involved in the modulation of diverse cellular events such as remodeling of actin cytoskeleton, regulation of focal adhesion dynamics, and cell migration. However, the cellular defects associated with βPix null condition have not been reported yet. To investigate the role of βPix in vivo, we have generated βPix knock-out mouse. βPix null (βPix<sup>−/−</sup>) mouse was early embryonic lethal at E8.5 due to the defects in placenta formation. So we established MEF(mouse embryonic fibroblast) cell lines from E8.5 βPix<sup>−/−</sup> mouse embryos by immortalization of MEFs with retroviral infection of SV40 large T antigen. βPix<sup>−/−</sup> MEFs show no βPix expression, but the expression of Rac1, Pak1, and Git1, which are well-known βPix-associated proteins, were normal. And βPix<sup>−/−</sup> MEFs are smaller than βPix<sup>+/+</sup> MEFs. Also in wound healing assay, the
migration pattern of βPix−/− MEFs seems non-directional and random. It is known that PDGF induces dorsal ruffles in fibroblasts. βPix−/− MEFs showed severe defects in dorsal ruffle formation, while βPix+/+ MEFs show normal dorsal ruffle formation. These results suggest that βPix is essential for directional migration and also for the PDGF-induced dorsal ruffle formation in fibroblasts. This study also shows that βPix−/− MEFs can be a valuable tool to study βPix function in diverse cellular events.

1154/B312
The Dvl-Associating Protein Daple Regulates the Activation of Rac and the Remodeling of Actin Cytoskeleton.
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Dishevelled (Dvl) is a multifunctional scaffold protein, which locates at the crossroad of two major Wnt signaling pathways, the canonical beta-catenin and the non-canonical planar cell polarity pathways. In the non-canonical pathway, the Rho family of small GTPases acting downstream of Dvl, plays important roles in the polarized reorganization of the actin cytoskeleton, which ultimately leads to changes of cell shape and motility. However, the mechanism by which Dvl regulates the activation of the small GTPases has not been elucidated. In this study, we show that Daple (Dvl-associating protein with a high frequency of leucine residues) promotes the activation of Rac through the interaction with Dvl. In HEK293T cells, overexpression of Daple induced the activation of Rac1, but not that of RhoA or Cdc42. In contrast, a deletion mutant of Daple, which lacks Dvl-binding three amino acids (Gly-Cys-Val) at its C-terminus, lost its ability to activate Rac1. The Daple-induced Rac1 activation was attenuated by knockdown of Dvl, suggesting either that Daple acts upstream of Dvl or that the Dvl/Daple protein complex is indispensable for Rac1 activation. Finally, we found that Daple is necessary for cell spreading and the formation of lamellipodial protrusion in fibroblasts. These data suggest that Daple mediates Dvl-dependent activation of Rac and the reorganization of actin cytoskeleton and identify the Dvl/Daple complex as a candidate mediator of Rac activation in the non-canonical pathway.

1155/B313
Role of Erk1/2 for Membrane Ruffle Formation and Chemotaxis of Microglia via the Regulation of Focal Adhesion.
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Microglia in the brain participate in the response to brain injuries and diseases in which their activation, migration, and proliferation are known to play crucial roles. Extracellular ADP has been reported to induce microglia chemotaxis and membrane ruffle formation through P2Y12 receptor. In this study, we examined the role of Erk1/2 in ADP-induced microglia chemotaxis and membrane ruffle formation. ADP stimulation transiently increased intracellular cAMP concentration, leading to the activation of Erk1/2 via Epac-Rap activation. Inhibition of Erk causes a significant reduction of the retraction of membrane ruffles and chemotaxis. In U0126-treated cells, paxillin remained at the membrane cortex and size and number of mature focal adhesions was not increased while most paxillin staining was associated with focal adhesions in 8-CPT-cAMP-treated cells. This result indicates that Erk1/2 activity might be required for the transformation of focal complexes into focal adhesions. Phosphorylation of Ser83 of paxillin was initially decreased below the basal level upon ADP stimulation and then started to increase after 2 min, which is similar to the activation kinetics of Erk1/2. Our results suggest that paxillin phosphorylation by Erk1/2 plays an important role in membrane ruffle formation and chemotaxis via the regulation of focal adhesion formation/maturation.
1156/B314
Rhomboid Family Protease RHBDL2 Catalyzes the Release of Thrombomodulin from the Surface of Keratinocytes and Promotes Cell Mobility.
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Thrombomodulin (TM) is a multifunctional transmembrane protein expressed on the surface of epithelial and endothelial cells. It has been reported that rhomboid family rhomboid-like-2 (RHBDL2) can specifically cleave the transmembrane domain of TM in the TM & RHBDL2 double transfected cells. We demonstrated that the expression of RHBDL2 is up-regulated in scratch-wounded culture of keratinocytes, and the rhomboid protease is involved in the modulation of TM shedding. The Western blot analysis indicated a positive relationship between number of the lines of scratched-wound and expression of RHBDL2 in human epithelial HaCaT cells. The amount of soluble TM (sTM) in the medium also increased in the same pattern. On the other hand, serine protease inhibitor, 3,4-dichloroisocoumarin (3,4-DCI), and RHBDL2 specific shRNA (shRHBDL2) reduce the wound-induced TM ectodomain shedding. The closure of scratched-wound was found to be significantly delayed in shRHBDL2 stable transfected cells. The conditioned medium from culture of scratch-wounded HaCaT cells and recombinant soluble TM enhanced wound closure of the HaCaT cell culture. The effect of the conditioned medium is significantly suppressed by the antibody against TM. Moreover the conditioned-medium from shRHBDL2 transfected cells does not enhance wound closure. In summary, the results suggest that RHBDL2 plays a critical role in releasing of TM from the keratinocytes to the medium, which in turn promote closure of scratched wound in the culture of keratinocytes.

1157/B315
A Novel Heat Shock Protein-90 (Hsp90) Autocrine Mechanism That Mediates Hypoxia-Driven Cell Migration during Skin Wounded Repair.
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Hypoxia plays a critical role in the pathogenesis of a variety of human diseases such as ischemic cardiovascular disease and cancer. After an acute skin injury, the microenvironment is extremely hypoxic due to the clotting of blood vessels and high oxygen consumption by cells in and around the wound site. We have shown that acute hypoxia is a potent stimulus to increase the migration of both epidermal and dermal cells. However, the mechanisms by which acute hypoxia promotes cell motility were not well understood. The purpose of this study was to elucidate the mechanism of hypoxia-driven human keratinocyte (HK) migration. We found that hypoxia, via the hypoxia inducible factor 1 (HIF-1) signaling, induces HKs to secrete heat shock protein 90-alpha (hsp90α) which, in turn, binds to the LDL Receptor-Related Protein-1 (LRP1) receptor on the HK surface and stimulates HK migration using an autocrine loop. Expression of a constitutively activated HIF-1 in HKs by lentiviral infection fully mimicked the hypoxia-driven motility in HKs even under normoxia. In contrast, expression of a dominant negative HIF-1 or siRNA down-regulation of HIF-1 completely abolished the hypoxia-driven motility. The exogenous addition of recombinant hsp90α to normoxic HK motility assays fully duplicated the pro-motility effect of hypoxia. The hypoxia-HIF-1 pathway-induced hsp90α secretion required no changes in the steady-state mRNA level or in the promoter activity of human hsp90α. However, inhibition of either the exosomal protein trafficking or the extracellular hsp90α blocked hypoxia-induced and HIF-1-driven HK migration. Hsp90α directly binds to the LRP-1 in HKs and genetic silencing LRP-1 completely blocked hypoxia-triggered HKC migration. Most importantly, topical application of recombinant hsp90α accelerated wound healing by increased re-epithelialization process in mice. In parallel, the FDA-approved RegranexTM (PDGF-BB) showed much less effect than recombinant hsp90α. This study has unveiled a previously unrecognized mechanism of hypoxia > HIF-1 > hsp90α secretion > LRP1 receptor > skin cell migration > wound healing and identified recombinant hsp90α as a potentially novel wound healing agent.
Role of the Cytoplasmic Domain of Coxsackie and Adenovirus Receptor (CAR) in Cell Migration.

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The Coxsackie and Adenovirus Receptor (CAR) is a cell adhesion molecule of the immunoglobulin superfamily. Low or absent CAR expression is seen in many primary tumors in comparison to adjacent normal tissue. CAR is also down regulated in many tumor cell lines and its re-expression inhibits growth of these cell lines. We have previously shown that CAR may affect cell migration, a priming step for cancer development, through its direct interaction with microtubules and actin, and that the cytoplasmic domain of CAR is required for decreased invasion and intracerebral growth of human glioma xenografts. To further study the role of the cytoplasmic domain of CAR in cell migration, we have begun to map the binding sites for the cytoskeletal proteins by cloning truncated versions of CAR that lack different domains of the C-terminal portion, followed by stable expression in glioma cells and evaluation of cell migration ability. In parallel, we are also examining the contribution of the four conserved tyrosines that are present in the cytoplasmic tail of CAR by mutating each individually, and in combination, to alanine residues. In cell migration assays, the single mutation of tyrosine 294 (Y294A) abrogated CAR-mediated inhibition of migration while the single mutation of tyrosine 313 (Y313A) had no such effect, demonstrating similar migration rates as those of cells expressing wild-type CAR. These studies will shed new light on the mechanism by which CAR may regulate tumour cell migration, and eventually, cell invasion and growth.

Altering RhoA and RhoC Expression Levels Causes Changes in Migration Properties of Epithelial Cells during Wound Closure.

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During metastasis, a subset of cells undergoes epithelial to mesenchymal transition where they acquire the ability to migrate away from the primary tumor. Rho GTPases are a family of signaling proteins involved in cell motility and are known to be up-regulated in various cancers. One member of this family, RhoC, is over-expressed in highly metastatic carcinomas while the RhoA isoform is not, despite sharing a 94% amino acid identity. To elucidate the different functions of RhoA and RhoC that implicate RhoC’s role in metastasis, we created stable PtK1 cell lines expressing fluorescent probes for both RhoA and RhoC and subjected them to a wound healing assay in order to stimulate directed migration. Measuring the change of area over time showed that RhoC cells migrate 1.5 times the rate of RhoA cells. In addition, we used single cell tracking software to determine migration rates, fluorescence intensity, and paths amongst individual cells at various points within the wound and monolayer to better clarify the differences in motility output of these cells. Western Blots were then performed to determine the amount of over-expression present in these cell lines. Interestingly, endogenous RhoC was decreased in both cell lines, while endogenous amounts of RhoA remained unchanged compared to controls. Because Rho GTPases are known to signal into focal adhesions, levels of adhesion expression were determined by looking at amounts of total and phosphorylated Paxillin as a possible explanation for the observed variation in migration rates. Western Blots showed that levels of total Paxillin and phosphorylated Paxillin were elevated in both cell lines when compared to controls, suggesting that subtle changes in over-expression are still able to promote a significant change in downstream targets within the cell. Together these preliminary results suggest that RhoA and RhoC isoforms may signal into distinct pathways.

Roles of KASH/SUN Nuclear Envelope Proteins SUN1/2 and Syne-1/2/Nesprin-1/2 during Neurogenesis and Neuronal Cell Migration.
How the cell positioning its nuclei is an important and interesting question during many biological processes. Recently studies have indicated the roles of the KASH-SUN nuclear envelope (NE) complexes in a number of cellular processes including nuclear positioning. We have taken a genetic approach to analyze the functions of SUN-domain proteins SUN1 and SUN2 and the KASH-domain proteins Syne-1/Nesprin-1 and Syne-2/Nesprin-2 in mice. Previously, we have demonstrated their roles in meiosis and myonuclear anchorage. Here we will report their functions in neurogenesis and neuronal migration, two fundamental aspects of mammalian brain development. While a number of cytoplasmic proteins, including dynein and Lis1, are known for their roles in connecting microtubules to the nucleus during interkinetic nuclear migration and nucleokinesis, the factors connecting dynein/Lis1 to the nuclear envelope (NE) remain to be determined. We found that Syne-1/2 and SUN1/2 double mutant mice display severe defects in multiple brain regions. Additionally, although Syne-2 single mutant mice are viable, they display severe defects in learning and memory. Our detailed analysis using various labeling methods indicated that in the cerebral cortex, SUN1 and SUN2 redundantly form complexes with Syne-2 to mediate the centrosome-nucleus coupling during both interkinetic nuclear migration and radial neuronal migration. We also provide biochemical evidence that Syne-2 interacts with both dynein/dynactin and kinesin complexes for its connection to the centrosome. Therefore, Syne-2 and SUN proteins mediate the pulling/pushing forces from dynein and kinesin to the NE during nuclear movement relatively to the cell body. These results fill an important gap in our understanding of mechanism of nuclear movement during neurogenesis and neuronal migration.

1161/B319
Functions of the mRNA Binding Protein, ZBP1, and Its Implications on Cell Motility.
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Post-transcriptional regulation of mRNA acts to control the expression level and location of specific proteins within cells, thereby inducing key responses in development, cellular differentiation, cancer progression and metastasis. The zipcode binding protein (ZBP1) acts to regulate β-actin mRNA translation through direct binding to the 3'UTR. Translocation of the ZBP1-RNA granule ensures proper localization of RNA to fibroblast lamellipodia, a process known to be critical for persistent cell motility. Active Src within cellular protrusions has been proposed to phosphorylate ZBP1 (at Y396) and activate translation of β-actin mRNA. Therefore we hypothesize that inhibiting this regulatory mechanism of ZBP1 will compromise a cell’s ability to spatially control β-actin mRNA translation. Through multivariate analysis of single cell-motility we show that transient expression of a phosphonull ZBP1 mutant (Y396F) significantly inhibits the cell’s ability to move in a persistent direction. These results suggest that average velocity and total path distance of chicken embryo fibroblasts expressing the mutant ZBP1 is unaffected, but the directional persistence of motion is significantly decreased. Additionally, derivation of mouse embryo fibroblasts (MEFs) from E14.5 ZBP1 knockout (KO) embryos has allowed us to further probe the importance of ZBP1 (also termed mouse IGF-II mRNA binding protein 1 or IMP1). ZBP1 KO MEFs show a less distinct defect in motility but have upregulated a ZBP1 paralogue IMP2 (mouse IGFII mRNA binding protein 2) which may act to compensate for the loss of ZBP1 and therefore abrogate a major motility defect. This evidence therefore suggests that the functional mechanisms of ZBP1 and its respective paralogues are implicated in a cell’s ability to move in a persistent direction.

1162/B320
The Transcription-Migration Interface in Heart Precursors of Ciona intestinalis.
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Gene regulatory networks direct the progressive determination of cell fate during embryogenesis, but how they control cell behavior during morphogenesis remains largely elusive. We investigated this problem using the migrating heart precursors of the ascidian Ciona intestinalis as a simple model. The ascidian embryos the B7.5 lineage comprises the trunk ventral cells (TVCs), which constitute the heart precursor cells that migrate into the trunk, and there sister cells that remain in the tail where they form anterior tail muscle cells (ATMs). Previous studies showed that heart specification and TVC migration require the transcriptional activities of the Mesp bHLH transcription factor and an Ets1/2-mediated FGF signal. The forkhead factor FoxF is up-regulated in TVCs downstream of Mesp and FGF signaling and is required predominantly for cell migration. We developed a method based on fluorescence activated cell sorting (FACS) and whole genome profiling of B7.5 lineage cells (TVCs and ATMs) following target molecular manipulations of Mesp, FGF signaling and FoxF to analyze gene expression changes that correlate with cell migration\textsuperscript{1}. We identified 130 genes whose expression correlate with cell migration. These genes belong to a broad range of functional and molecular classes and further analysis indicated that the heart network regulates genes involved in most cellular activities required for migration, including adhesion, cell polarity, and membrane protrusions. Only a fraction of the genes required for each one of these activities appeared regulated. We demonstrated that FGF signaling and FoxF directly up-regulate the small GTPase RhoDF, which synergizes with constitutively expressed Cdc42 to contribute to the protrusive activity of migrating cells. Moreover, RhoDF induces membrane protrusions independently of other cellular activities required for migration. We propose that transcription regulation of specific effector genes determines the coordinated deployment of discrete cellular modules underlying migration. 1. Christiaen, L., Davidson, B., Kawashima, T., Powell, W., Nolla, H., Vranizan, K. & Levine, M. Science 320, 1349-52 (2008).

1163/B321

\textbf{Cytoplasmic Dynein and a Nonconventional Kinesin Drive Interkinetic Nuclear Migration in Neuroepithelial Progenitor Cells.}

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Extensive and precisely executed neuronal migration is essential for normal brain development. This complex process is initiated in a neuroepithelium of highly-elongated cells (aka radial glia), which give rise to neurons, glia, and adult neural stem cells. The radial glial cell nuclei exhibit a remarkable series of long-range, cell cycle-dependent oscillations, known as "interkinetic" nuclear migration, with mitosis occurring at the ventricular (apical) cell terminus, and S-phase occurring basally (away from the ventricular surface). The mechanism and purpose of this behavior has remained mysterious for the past half century. We have been studying neuronal migration using in utero electroporation of E16 rat brain with shRNA-encoding cDNAs along with markers for subcellular structures. By fixed and live imaging of brain slices we found that knockdown of the lissencephaly gene, LIS1, blocks neuronal migration, including effects on the interkinetic nuclear oscillations (J Cell Biol, 176:935; Nat Neurosci, 10:970). We have now investigated radial glial cell behavior in detail. Using CFP-histone H1 we find that apically- and basally-directed nuclear migration in these cells are each unidirectional, but differ in character: apically-directed movement is fast and highly discontinuous, whereas basally-directed movement is very slow and continuous. Using DsRed-centrin, we find that nuclei migrate completely without associated centrosomes, which remain at the ventricular surface throughout the nuclear oscillatory cycle. Using GFP-EB3 we find microtubules to be uniformly oriented with their plus ends pointed basally throughout the 1-2 mm length of the radial glial cell. Dynein heavy chain RNAi inhibited apically-directed nuclear movement. Blebbistatin and Myosin II RNAi had no effect. An RNAi screen for the basally-directed motor identified a nonconventional kinesin, knockdown of which specifically blocks basally-directed nuclear migration. We propose that interkinetic nuclear migration involves a cell

**1164/B322**

*Laser Ablation of the Centriole in Migrating Cells Leads to a Quantifiable Change in Microtubule Organization.*

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Cell migration is necessary for biological processes such as wound-healing and embryonic development and plays a key role in pathologies such as metastasis and atherosclerosis. When a cell receives stimuli to migrate, it polarizes by concentrating signaling molecules and components of the cytoskeleton to the leading edge of the cell. In its polarized state, F-actin and microtubules are concentrated at the front of the cell forming the lamellae. In this study we use a near infrared (780 nm) 200 femtosecond laser to ablate the centriole in migrating cells. In addition, we have developed a method to quantify the microtubule network as a way to distinguish changes in the cells’ cytoskeleton in response to centriole ablation. Quantification of the polarization process was conducted on several cell types including PTK2, fish keratocytes, U2OS and IMR90 cells. The quantification method was also tested on treated cells with cytochalasin and wortmannin to test its ability to differentiate between polarized and nonpolarized cells. We calculated the position of the microtubule tip center of mass by determining the average pixel positions of all visible microtubule tips at the periphery of the cell and determined changes relative to the lamellae and nucleus centroid. Initial results show a significant change in the microtubule mass between polarized and nonpolarized cells. This corresponds to the asymmetric shift of the microtubule network in the direction of the cell migration. Destabilization of microtubules and actin in both PTK2 and keratocyte cells leads to a quantifiable nonpolarized cell morphology. The quantification method was applied to migrating cells in which the centriole was laser-ablated. A significant difference (p = 0.02) in the microtubule cytoskeleton was observed between the control and centrosome-deficient cells. Supported by the Air Force Office of Scientific Research # FA9550-08-1-0384

**Organogenesis (1165 – 1185)**

**1165/B323**

*Wnt5a Is Required for Epithelial Cell Polarity, Proper Lumen Formation, and Axis of Cell Division.*

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Objective. Cell polarity and cell division are important events during gut organogenesis as the intestinal epithelium undergoes a dramatic morphological re-organization designed to create villi and maximize the surface area for absorption. Starting at mouse embryonic day (E)13.5, a period of proliferation expands the epithelial cell layers, epithelial polarity creates an apical surface and expands the lumen, and dynamic cell movements lengthen the anterior-posterior axis of the gut and form radially projecting villi. We report the dynamic states of cell polarity, cell division, lumen formation, and cell movements in the intestinal epithelium and analyze the role of Wnt5a in controlling these processes in vivo. Methods. Proliferation was investigated by Ki67 staining; DNA synthesis was examined by BrdU incorporation; mitosis was assessed by Phospho-Histone H3 staining; and cell polarity markers were examined during intestinal epithelial rearrangement in E14.5-E16.5 embryos from WT and Wnt5a-/- mice. for dividing cells, the axis of cell division relative to forming secondary lumina was assessed. Results. Ki67 staining revealed that almost all mouse intestinal epithelial cells are cycling (S, G2, M) at E14. Examination of the labeled pattern 15 minutes after a BrdU pulse revealed that S-phase cells of the stratified gut epithelium
are almost exclusively attached to the basement membrane. Moreover, very few mitotic figures were seen, of which 95% are located near the luminal surface of the multi-layered epithelium, suggesting a link between division and lumen formation. We found that apical surfaces were present in both WT and Wnt5a, but secondary lumina appeared reduced and disorganized in Wnt5a-/- intestines. Aberrant cell division axes, vacuole accumulation, and mislocalized Golgi were confirmed at E14.5 in Wnt5a-/- epithelia, suggesting improper trafficking of apical membrane in Wnt5a intestines. Conclusions. Intestinal epithelial cells synthesize DNA when attached to the basement membrane and move toward the luminal region before undergoing division. We hypothesize that cell division and creation of the apical surface during lumen expansion are intimately associated and controlled by Wnt5a.

1166/B324
Downstream Non-Canonical Wnt Components Are Required for Epithelial Tubulogenesis in the Xenopus Pronephros.
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Wnt signaling is essential in embryonic axis formation, body patterning and organogenesis. Wnt pathways involve binding of extracellular Wnt ligands to transmembrane receptors, activating “canonical” and/or “non-canonical” signaling trajectories. Renal tubule formation in amphibians and mammals requires Wnt pathway activation, and recent work from both our group and others indicates that canonical/β-catenin signaling is essential. Non-canonical pathways contribute to normal processes such as cell polarization and cytoskeletal control, and while Wnt9b involvement in kidney tubulogenesis has recently been shown, the specifics of the pathways involved remain in question. A further incentive to address these mechanisms is that pathological deficiencies in non-canonical Wnt signaling that are thought to contribute to developmental defects such as polycystic kidney disease and nephronophthisis. Here, using the amphibian Xenopus laevis, we have begun to test the roles of non-canonical Wnt signals in kidney tubule morphogenesis. Xenopus offers experimental advantages including the facile introduction of exogenous constructs to block or activate signaling pathways, rapid development and easy visualization of the forming kidney immediately beneath the lateral surface ectoderm. The roles of two planar cell polarity branches of the non-canonical Wnt pathways, namely the Rac1/JNK trajectory and the Rho/Daam1 trajectory, are being assessed. We find that incubation of post-gastrula embryos with the JNK chemical inhibitor SP600125 consistently resulted in tubule dysmorphogenesis. Likewise, tubulogenesis is reduced using the targeted pronephric expression of a previously characterized dominant inhibitor of the Rho PCP pathway (N-Daam1) or morpholino antisense oligonucleotides to knockdown Daam1 expression. Daam1, a formin protein, may contribute to tubulogenic morphogenesis via its regulation of the actin cytoskeleton. Our very preliminary data thus suggest that both the Rac1/JNK and Rho/Daam1 planar cell polarity (PCP) trajectories are contributory.

1167/B325
Integration of TGFβ Signals Directs Asymmetric Morphogenesis of the Zebrafish Heart.
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The Nodal signaling pathway plays a conserved role in establishing differences between the left and right sides of all vertebrate embryos. This initial patterning along the left-right axis is required for the subsequent asymmetric positioning and morphogenesis of the visceral organs. Recent work has increased our understanding of these events; however, the mechanisms by which
asymmetric expression of Nodals and other TGFβ signals instruct the movements of cells and tissues remain largely unidentified. We are using zebrafish cardiac development as a model to investigate the requirements for TGFβ signaling in directing asymmetric morphogenesis. Through time lapse analysis of myocardial cell migration and careful comparisons of laterality phenotypes in a number of conditions in which TGFβ signaling is altered or inhibited, we are addressing how individual signaling pathways influence cardiac laterality. Additionally, we wish to examine how multiple TGFβ signals are integrated to produce a consistent morphological response to asymmetric information established at the molecular level. We have found that the laterality of Nodal signaling influences the first asymmetric evident in the heart; a left-directed migration of myocardial cells within the cardiac cone. Interestingly, we have discovered that a combination of Nodal and BMP signaling in the cardiac region is required for proper morphogenesis to occur. While loss of one or the other of these pathways results in randomization of cardiac asymmetries, absence of both TGFβ signals leads predominantly to a loss of asymmetric morphogenesis. Further investigation of the specific requirements for these pathways will provide new insights into how TGFβ signaling is regulated and integrated within the heart to promote consistent asymmetries in myocardial cell migration and overall cardiac laterality determination.

1168/B326

The Mouse Mammary Microenvironment Redirects Mesoderm-Derived Adult Marrow Cells to a Mammary Epithelial Progenitor Cell Fate.

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Previous studies have identified a lobule-limited, pluripotent mammary epithelial progenitor that is identified in post-parous mammary gland by the conditional activation of β-galactosidase (βgal) from a ubiquitous promoter (Rosa26) via whey acidic protein promoter (WAP)-driven Cre-lox recombination. Spermatogenic and neural stem cells, carrying these reporters, are re-directed to mammary epithelial progenitor cell fates in the presence of wild type mammary epithelial cells during regenerative mammary outgrowth in epithelium-cleared fat pads. Surviving βgal + cells in post-lactation, involuted glands indicates progeny from the non-mammary donor. Testicular and neural stem cells arise from the embryonal ectoderm. To test whether mesoderm-derived stem cells could respond to mammary epithelial-specific signals and adopt ectodermal cell fates, we mixed FACS-purified Lin-, cKit+ adult male bone marrow cells with equal numbers of mammary epithelial cells and implanted the mixture into cleared mammary fat pads of immuno-compromised female hosts. Following pregnancy, lactation and involution in the recipient females, the resulting outgrowths were examined for βgal-expressing cells. Robust contribution of βgal + epithelial progeny from bone marrow donor cells was observed in the resulting chimeras. FISH for the Y chromosome in mammary cells demonstrated the male donor origin of the βgal+ cells. Co-expression of mammary epithelial-specific markers and βgal demonstrated that the non-mammary donor cell progeny had adopted bona fide mammary epithelial cell phenotypes. The βgal + cells were found both in luminal and basal mammary epithelial cell types and the former were shown to be capable of milk protein synthesis in pregnant recipients. The bone marrow-derived progeny exhibited properties consistent with those previously demonstrated for parity-identified lobule-limited epithelial progenitor cells. The male βgal + cells were capable of symmetric expansion and retention of their pluripotent nature over three serial passages. Our evidence shows that the signals provided by the mammary microenvironment are capable of redirecting mesoderm-derived adult bone marrow-derived cells to produce mammary epithelial cell progeny.

1169/B327

C. elegans Septins Function in Arcade Cell Polarization during Pharyngeal Morphogenesis.

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Septin family GTPases are involved in cytokinesis in animals and fungi, but their expression in post-mitotic cells suggests that they also have non-mitotic functions. The nematode Caenorhabditis elegans is ideal for studying basic, conserved functions of septins, as there are only two worm septin genes, unc-59 and unc-61, the fewest found in any organism. We find that mutations in either or both of the C. elegans septins result in considerable lethality in the first larval stage. This larval lethality results from malformation of the pharynx, which fails to attach to the mouth. The nematode pharynx is an organ comprised of muscles, epithelial cells and neurons that functions to ingest, concentrate and process food before it is pumped into the intestine for digestion. Larvae with unattached pharynges are unable to feed and starve to death. Pharyngeal markers are normally expressed in larvae with unattached pharynges, suggesting that all of the normal types of pharyngeal cells are present. The unattached pharynges also have normal numbers of pharyngeal nuclei, indicating that the defects are likely to result from morphogenetic failures, rather than from failed cell divisions. The apical junction marker AJM-1 fails to concentrate in the apical domain of the arcade cells, which mediate attachment of the pharynx to the mouth. This suggests that pharyngeal attachment fails in the septin mutants at or prior to arcade cell epithelialization. When pharyngeal attachment fails, the arcade cells also fail to localize two other apical markers, PAR-3 and PKC-3, and the normal apical enrichment of actin filaments and microtubules in the arcade cells does not occur. These results suggest that arcade cell apical polarity and cytoskeletal defects underlie the septin mutants’ pharyngeal attachment failures. We hypothesize that septins function in organization of the arcade cell apical cytoskeleton, which is necessary for their accurate polarization and epithelialization.

1170/B328
The Hippo Pathway in Neural Development: Orchestrating the Survival, Proliferation, and Fate Choice of Neural Progenitor Cells.
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The Hippo pathway is a critical regulator of tissue growth in Drosophila. It operates through a kinase cascade that culminates in the phosphorylation and inhibition of the transcriptional coactivator Yki/YAP. Inactivation of the pathway results in the accumulation of unphosphorylated Yki/YAP, which interacts with the TEA-domain transcription factor TEAD and activates genes involved in cell proliferation and survival. Although most components of this pathway are conserved in vertebrates, its role during vertebrate development has not been well studied. The objective of our study is to determine the function of the Hippo pathway during vertebrate neural development, especially its function in neural progenitor cells. Using the chick neural tube as an In Vivo model system, we analyzed the core components of the pathway through gain-of-function and loss-of-function experiments. We found that YAP and TEAD gain-of-function causes a marked expansion of the neural progenitor population. Their loss-of-function results in increased cell death. Actively repressing their downstream genes leads to premature neuronal differentiation. Inhibiting the upstream kinases of the pathway also causes neural progenitor overproliferation. Part of these findings has also been confirmed during mouse neural development. Thus, the Hippo pathway regulates the growth of the vertebrate nervous system by affecting the survival, proliferation, and fate choice of neural progenitor cells.

1171/B329
Smoothened Controls Cell-Cell Contact Remodeling during Single Lumen Formation in the Zebrafish Gut.
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Single lumen formation is a fundamental aspect of tubulogenesis. We use the zebrafish gut as a model to investigate lumen formation. Early in development the zebrafish gut is a tube with multiple small lumens, which eventually coalesce into a single lumen. We have previously shown
that fluid accumulation is required for the coalescence of multiple small lumens into one. However, other processes such as contact remodeling must also take place to ensure proper lumen formation. Here we show that zebrafish mutants for *smo* fail to form a single lumen in the gut and instead form two large lumens. Our data suggest that this phenotype is due to a defect in cell-cell contact remodeling. We identified two genes regulated by *smo* signaling, a Na/H\(^+\) exchanger (NHE) and an intracellular chloride channel (CLIC). Knockdown of these channels also results in a double lumen phenotype. Transport of Na\(^+\) and Cl\(^-\) ions is important in regulating organelle acidification, a process required for sorting and recycling which facilitates cell-cell contact remodeling. Furthermore, inhibition of acidification results in a double lumen phenotype. Our data suggest that NHE and CLIC function in part in organelle acidification. Therefore we conclude that *smo* controls cell-cell contact remodeling during lumen formation at least in part by regulating Cl\(^-\) and Na\(^+\) ion channels that function in endosome acidification.

1172/B330
dWee1 Is Essential for G2/M Timing Whereas dMyt1 Maintains Cytoskeletal Structures during *Drosophila* Spermatogenesis.

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The G2/M transition is considered an "all or none switch", with cells proceeding inexorably from G2 to M phase due to self-amplifying Cdk1 activation feedback mechanisms. Our main objective was to test the "all-or-none" model in an In Vivo system. To test this hypothesis we focused at 90 hour long prolonged G2 phase arrest in the 16-cell primary spermatocytes during Drosophila male meiosis and determined how Cdk1 inhibitory kinases (dWee1 and dMyt1) affect Drosophila spermatocyte pre-meiotic G2 arrest. By correlating the position of PH3-labeled meiotically dividing spermatocytes from the apical tip of the testis with spermatocyte cell growth using live cell imaging and Cyclin a localization followed by quantification and statistical analysis, we observed that loss of dMyt1 does not affect the normal meiotic G2/M timing whereas loss of dWee1 results in premature meiotic G2/M transition. However, the myt1 mutant spermatocytes exhibited profound defects in the structure of membrane-bound organelles (fusomes and Golgi), as well as premature segregation of centrosomes, disengagement of centrioles and formation of multipolar spindles. From these results we conclude that dWee1 inhibition of nuclear Cdk1/Cyclin is critical for regulating G2/M timing; whereas dMyt1 activity is important for assembly or maintenance of cytoplasmic structures. These conclusions do not support a literal interpretation of the "all-or-none" model for Cdk1 activation at the G2/M transition. Instead, they provide first In Vivo evidence that dMyt1 and dWee1 serve specialized, spatially distinct novel Cdk1 regulatory functions important for coordinating cell cycle progression with developmental processes.

1173/B331
Development of Gastric Cell Lineages in Neonatal Rabbits.

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The morphology of adult gastric mucosa has been well characterized in terms of cell types and their localization. Surface mucous cells line the mucosal surface, forming pits that extend to the glands. The neck of the gland contains mucous neck cells and parietal cells. Parietal cells and chief cells make up the base of the gland, with parietal cells predominating nearer the neck and chief cells predominating toward the base. Previous studies have created a model for the constant renewal of the gastric epithelium by differentiating from stem cells located in the isthmus between the pit and neck. Here we report the morphology and development of cell lineages in neonatal rabbits (3-57 days post partum) using immunohistochemistry to identify the appearance of specific markers and cells types. In very young animals (3-5 d) the mucosa consists of a thin layer of columnar epithelial cells, with a globular arrangement of pre glandular and mesenchymal cells beneath the surface layer. Clear glandular morphology was apparent at ~9 d in a total mucosal thickness of ~200 µm, which progressed over the next 3 wks to well developed and
organized glands (mucosal thickness ~700 µm). Parietal cells (H,K-ATPase; HK) were present at the earliest times, with increasing numbers and density over the first 3 wks, though the intracellular organization of HK was not as developed as in older animals. These observations are consistent with biochemical studies in early rabbit neonates, where HK was found in a microsomal fraction that lacked critical properties of mature HK-rich vesicles. Ezrin was evident in the earliest parietal cells and in lesser amounts in other epithelial cells, in contrast to the adult where ezrin is exclusive to parietal cells. Neck cells were present at the earliest ages, however, chief cells were not apparent in early neonates, but appeared in older animals. These data suggest distinct development of gastric cell lineages.

1174/B332
Hand2 Insures an Appropriate Environment for Cardiac Fusion by Limiting Fibronectin Levels.
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Interactions between cells and their environment are fundamental for establishing tissue organization, yet the mechanisms controlling these interactions are poorly understood. Here we show that the myocardial transcription factor Hand2 plays a crucial role in controlling the environment that facilitates cardiac morphogenesis. Heart tube formation requires the fusion of bilateral cardiomyocyte populations as they move toward the embryonic midline. Prior work has established that Hand2 is essential for cardiac fusion, but these studies have not revealed the mechanisms by which Hand2 regulates cardiomyocyte behavior. Although hand2 is expressed in cardiomyocytes, mosaic analysis demonstrates that hand2 plays a non-autonomous role in regulating cardiomyocyte movement, suggesting that downstream components of the Hand2 pathway influence the cardiomyocytes’ environment. Gene expression profiling reveals heightened expression of fibronectin 1 (fn1) in hand2 mutant embryos, consistent with the increased deposition of Fn around hand2 mutant cardiomyocytes. These results suggest that the Hand2 pathway might promote cardiac fusion by limiting the levels of Fn in the extracellular environment. Indeed, we find that reduction of fn1 function enables rescue of cardiac fusion in hand2 mutants. Furthermore, analysis of the myocardial epithelial structure in the rescued hand2 mutants suggests that myocardial tissue organization can be achieved without complete epithelial polarization. Together, our data demonstrate that the Hand2 pathway regulates the tissue-environment interactions necessary for cardiac fusion through modulation of Fn1 levels.

1175/B333
Endogenous Patterns of Mechanical Stress Regulate Mammary Branching Morphogenesis.
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How do branched organs obtain their unique tree-like shapes? Patterning of branching requires local regulators to establish regional differences within the developing tissue. In addition to biochemical signals, cells within tissues experience endogenous and exogenous mechanical stresses. We numerically predicted and experimentally confirmed that mechanical stress is non-uniformly distributed across three-dimensional (3D) tissues. The stress profile is governed by cellular contractility, extracellular matrix (ECM) stiffness, intercellular cohesion and tissue geometry. Using a lithography-based 3D culture model we identified endogenous mechanical stress as an independent regulator of branching morphogenesis of mammary epithelial cells. Our findings suggest that the pattern and magnitude of mechanical stress control morphogenesis by specifying the pattern and extent of branching, respectively. These data provide understanding of the physical parameters that pattern developing epithelial tissues, and can help elucidate the mechanisms that lead to disruptions of the normal morphogenetic program in cancer.
Kif-K Regulates the Mesenchymal Condensation on Contact with Ureteric Buds in Kidney Development.
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The mammalian kidney, the metanephros, is formed by a reciprocally inductive interaction between two precursor tissues, the metanephric mesenchyme and the ureteric bud. On contact with ureteric buds, the mesenchymal cells condense around the ureteric bud. We previously demonstrated that Sall1, a zing finger protein expressed specifically in the mesenchyme, is essential for kidney development. To explore molecular cascades crucial for renal development, we performed microarray analysis using Sall1-positive mesenchymal cells. Kif-k, a kinesin family member, was found to be expressed abundantly in Sall1-positive metanephric mesenchyme. Here we show that targeted disruption of Kif-k causes kidney agenesis with the impaired ureteric bud attraction into mesenchyme. Furthermore, Kif-k null mice showed the impairment of mesenchymal condensation adjacent the ureteric bud, which presumably resulted in the failed maintenance of Gdnf, a critical ureteric bud attractant. Therefore, Kif-k regulates the mesenchymal condensation on contact with ureteric buds in kidney development.

Interdisciplinary and Systemic Analysis of Organogenesis In Vitro: 4D Analysis of Lumen-Forming MDCK Cell Aggregates.
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Organs are elaborate cell communities performing specialized functions. The cell as their smallest unit plays an important role in shaping organs. Therefore, the ability of a cell to control its own shape and contacts with its neighbors is central to organogenesis. Mammalian organs made of epithelial tissue such as kidney and lung consist of two building blocks, cysts and tubules. These are each characterized by a central volume, a lumen, surrounded by a monolayer of polarized cells. How is this space generated in the first place? Understanding lumen formation reflects understanding of how cells polarize during organogenesis and how regulation of cellular mechanics coordinates cell shape changes. We thus investigate how physical concepts influence biochemical processes. The development of single MDCK cells into aggregates with lumen analyzed by quantitative 4D imaging occurs in a sequential four-step process in the presence of aggregate rotation but in the absence of cell death or division. The organization of the major surrounding matrix fibers is not significantly affected during rotation, indicating that the forces exhibited by the aggregate during rotation are not sufficient to deflect the major fibers notably. Applying our newly developed mathematical method for the 3D analysis of rotation of deformable systems we discovered three types of rotational behavior during lumen initiation and a high variability of motility parameters indicating that it is a robust process. Opening up a lumen depends on targeted membrane traffic and two force generation mechanisms (relieve of ROCK dependent myosinII contraction and barrier enclosed water influx) that act in parallel. These mechanisms might either regulate or be regulated by the accompanying morphometric changes in cell volume and surface areas.

Role of Perk in the Dynamic Patterning of Stem Cells during Feather Morphogenesis.
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Understanding how homogeneous stem cells are organized into desired configurations and programmed toward specific fates is a fundamental issue in developmental biology and regenerative medicine (Chuong and Richardson edit, 2009). We examine periodic patterning during feather primordia morphogenesis to analyze this process (Widelitz et al., 2006; Lin et al., 2006). Feather explants offer a testable model in which multi-potential cells are organized into hexagonally arranged primordia and inter-primordial space. We previously showed that FGF induces feather bud formation and helps to establish periodic patterning through a Turing reaction-diffusion system (Jung et al., 1998) but the molecular and cellular mechanisms underlying these processes remain unclear. Recently we showed how this chemical pattern is translated into a cellular configuration (Lin et al., 2009). Our study shows that chemotaxis toward a chemical signaling center requires the phosphorylation of MAPK/ERK. A localized source of FGF produced by reaction-diffusion is required for this phosphorylation. This finding is based on retroviral mediated expression of a soluble dominant negative FGFR1, application of a chemical ERK inhibitor (U0126), and siRNA mediated suppression of Raf. A U0126 dose response curve produced a continuum of feather patterns, ranging from stripes to large and small spots in a U0126 concentration- and tissue stage-dependent. We conclude that ERK activity-dependent mesenchymal cell chemotaxis is essential for converting micro-signaling centers into stable feather primordia. A mathematical model based on short-range activation, long-range inhibition, and cell chemotaxis is developed and shown to simulate observed experimental results. Thus we can re-pattern a group of stem cells into different periodic patterns by changing their microenvironment. It illustrates the pleomorphic nature of stem cells, and offers the ability to modulate them into desired configurations for tissue engineering (Chuong et al., 2005). Dynamic cellular behavior, recorded by growing skin explants in a Wafergen, SmartSlide environmental chamber, will be shown and analyzed with an attempt to understand these seemingly simple but also complex events.

1179/B337
Convergent Extension Movements in Growth Plate Chondrocytes Require Gpi-Anchored Cell Surface Proteins.
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Endochondral bone growth is regulated by the rate of chondrocyte maturation in growth plate cartilage, an architecturally complex tissue containing highly ordered columns of proliferative chondrocytes. The relationship between growth properties of the cartilage and chondrocyte polarity and organization is not well understood. Previously, we showed that proliferative chondrocytes divide laterally then daughter cells intercalate into columns in processes dependent on noncanonical frizzled signaling. Here we show that loss of glycosylphosphatidylinositol (gpi)-anchored proteins interferes with cell intercalation without substantially affecting the division plane. Lateral, rather than longitudinal, expansion of clones is associated with altered dimensions of long bones consistent with the columnar arrangement of proliferative chondrocytes defining vectors of bone growth via the process of convergent extension. Interestingly, gpi-anchored proteins are also required for polarity of stereocilia and polarized distribution of the planar cell polarity protein Vangl2 in the inner ear. Together, these data suggest that distinct cell polarity events share common core components.

1180/B338
The Regulation of Bud and Duct Formation during Lacrimal Gland Branching Morphogenesis.
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The lacrimal gland is a classical example of a structure that develops through branching morphogenesis. This process involves determination of when and where a branch will occur and how long the tube (stalk) grows before branching occurs again. The epithelial bud initiation is
regulated by restricted expression of Fibroblast Growth Factor 10 (FGF10). However, factors involved in LG duct differentiation are still undefined. FGF10 belongs to FGF7 subfamily of FGFs that signal specifically through epithelially expressed FGFR2b. In many branched organs/tissues including the LG, submandibular gland (SMG) and lung, FGF10 has been shown to be expressed in a distinctive pattern at the tips of the buds. In contrast, FGF7 is uniformly distributed throughout the mesenchyme. Here we show that the isolated LG bud, exposed to FGF10 loaded on a bead, extends towards the bead and produces a structure without branches but with defined distal (bud) and proximal (duct) morphology. In contrast, beads loaded with FGF7 do not induce directional elongation of the lacrimal bud, instead inducing a local growth of the bud with no distinctive duct morphology. The examination of the gene expression profiles of LG explants treated with FGF10 or FGF7 using a RT PCR-array (focusing on genes within the MAP kinase signaling pathway) revealed that FGF10 application up-regulates expression of several genes involved in duct differentiation while FGF7 application did not. Thus, FGF10 has at least two distinctive functions during lacrimal gland development- regulating both LG bud induction and LG duct differentiation.

1181/B339
**Morphogenesis, Cell Motility and Polarity in the Developing Vertebrate Eye.**
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The vertebrate eye initially evaginates from the brain neuroepithelium, undergoing striking cell and tissue rearrangements to form the optic cup. Using zebrafish, we aim to develop a cellular and molecular understanding of the morphogenetic movements that generate the vertebrate optic cup, using three levels of analysis: tissue movements; single cell behaviors and polarity; and molecular mechanisms that control these movements. at the tissue level, we perform whole-eye timelapse imaging throughout the entire 12-hour period of eye morphogenesis; every cell is labeled for membranes (EGFP-CAAX) and chromatin (histone2A-mCherry). Using these 4D data sets and custom cell tracking software, we are generating a comprehensive map of cell movements and mitoses. Analysis has revealed large-scale tissue movements and an unexpected flow of cells between certain subregions of the developing eye. Furthermore, experiments with cell cycle inhibitors suggest that mitosis is dispensable for basic aspects of morphogenesis. Therefore, we focused on cell movements as the primary basis of optic cup formation. for single cell analysis, we utilize the photoactivatable fluorophore Kaede to observe cell behaviors throughout eye morphogenesis. Surprisingly, retinal progenitor cells display active lamellipodial protrusions, appearing migratory for most of the process before finally acquiring a stereotypical columnar epithelial morphology. Because these cells comprise an epithelial tissue, we examined cell polarity. Strikingly, markers of apicobasal polarity are localized as early as the onset of optic vesicle evagination; thus, the epithelium is polarized despite active motility of its component cells. To begin to uncover molecular pathways controlling eye morphogenesis, we are examining the role of laminin. Laminin is required for optic cup formation: in bashful (laminin-α1) mutants, failure of optic vesicle invagination leads to a protruding lens phenotype, and apicobasal polarity is disrupted. at the single cell level, retinal progenitors still have active protrusions and appear to migrate, but lose apicobasal register, leading to a prematurely multilayered retina. We are now identifying the laminin receptor.

1182/B340
**CMV-Induced Embryonic Cochlear Pathogenesis.**
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Congenital cytomegalovirus (CMV) infection is the most common non-genetic cause of sensorineural hearing loss (SNHL) in children, accounting for between 20 and 60% of all SNHL. It is estimated that at least 8000 infants born annually in the US will have congenital CMV-induced SNHL, with a significant proportion of these children exhibiting delayed onset and progressive deterioration of hearing after the newborn period. Currently, little is known about the mechanism...
underlying CMV-induced birth defects. Since in utero CMV infection causes SNHL whereas postnatal infection does not, we postulate that CMV-induced cochlear malformations are caused by viral-induced dysregulation of multiple host (embryonic) cell signaling pathways essential for normal cochlear morphogenesis and that the severity of dysmorphogenesis is time-dependent. We have developed a novel mouse embryonic organ culture model of CMV-induced cochlear malformations and demonstrate that active mouse CMV (mCMV) infection of embryonic mouse E15 cochleas in vitro induces cochlear dysmorphology and labyrinthitis, with abnormal infected and affected cells being seen in the organ of Corti, Reissner's membrane, stria vascularis, scala vestibule and scala tympani. Importantly, mCMV induces numerous supernumerary hair cells in a disorganized arrangement, as well as a dramatic and substantial change in cell proliferation. Using quantitative RT-PCR, we demonstrate that mCMV induced significant changes in the expression of molecules important to differentiation, particularly that of hair cells. Rescue with acyclovir indicates that mCMV replication is necessary to initiate and sustain progressive cochlear pathogenesis. Taken together, our data indicates that mCMV infection in vitro has a profound impact on embryonic cochlear differentiation. Our results also indicates that this embryonic mouse cochlear culture system models the inner ear pathology seen in children with congenital CMV infection and can be used to investigate the mechanism underlying CMV-induced cochlear pathogenesis in vivo.

1183/B341
Fibroblast Growth Factor Receptor 1 (FGFR1) Function in Inner Ear Hair Cell Development.
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The inner ear detects sound and balance by means of ciliated mechanoreceptors known as sensory hair cells (HC). Fibroblast growth factor (FGF) signaling plays recurring role in the development of the inner ear. FGF is involved in the early inductive and morphogenetic events (otocyst development) as well as the formation of the sensory epithelia that consists of HC and support cell (SC). Tissue-specific deletion of FGF receptor 1 (FGFR1) results in severe defects in the specification of the sensory epithelia (Pirvola et al., 2002). However, the later roles of FGF signaling in the HC are unknown. To ask whether FGFR1 functions after the specification of the sensory epithelia, we investigated the requirement for FGF signaling using SU5402, an FGFR inhibitor. We find that in chick the inhibition of FGFR from embryonic day 7 (E7) does not affect HC specification but causes a defect in the kinocilium, and perturbs planar cell polarity of HC. Immunostaining of FGFR1 in the sensory epithelia revealed that prior to E9, FGFR1 is expressed in both HC and SC. Intriguingly, in HC FGFR1 also distributes on the kinocilium, where the receptor is colocalized with the molecular motor KIF3A. After E9 FGFR1 expression increases, while the receptor expression becomes robust in HC. At this stage the receptor is translocated to the apical side of the HC and then localizes to the newly forming stereocilia. These data have begun to dissect a role for FGFR1 during later inner ear development and function.

1184/B342
Netrin1 Function in Morphogenesis of the Inner Ear.
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Netrin1 (Ntn1) is a key regulator during the development of the intricate three-dimensional structure of the inner ear. For example, Ntn1 mutant mice lack the proper formation of the vestibular semicircular canals, necessary for the sensation of balance and orientation. Vestibular canals develop by fusion or resorption of discrete regions of epithelial cells within the otic vesicle, termed the fusion plate. At the cellular level, this fusion event is accompanied by changes in cell morphology and breakdown of the basement membrane (BM) that normally separates the fusing epithelium from the surrounding mesenchyme. Disruption of the Lrig3 gene in mice results in ectopic and elevated expression of Ntn1 in the fusion plate. In contrast to the Ntn1 null mouse, ectopic Ntn1 expression leads to expanded BM breakdown, thinning of the otic epithelium, and
excess fusion, resulting in a truncation of the lateral semicircular canal. To determine whether ectopic expression of Ntn1 is sufficient to induce ectopic fusion and canal malformations, we used virus-mediated gene delivery. RCAS is a replication competent retroviral system based upon Rous sarcoma virus (RSV) that allows for sustained ectopic gene expression in the developing chick. We used RCAS to infect the chick otic vesicle and misexpress a secreted-GFP control or full-length Ntn1. Ntn1 is incorporated into the BM and causes a thinning of the epithelium typical of expanded fusion. However, the fusion plate cells fail clear properly, suggesting that excess Ntn1 in the chick inner ear reduces recruitment or resorption of fusion plate cells. This result demonstrates an uncharacterized role for Ntn1 in the prevention of cell resorption, possibly due to inhibition of recruitment or apoptosis during complex tissue morphogenesis. N- or C-terminal truncations of Ntn1 will be assessed in the chick model to determine which domains mediate effects on cell viability and BM incorporation.

1185/B343
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Cell migrations are vital for the development of multicellular organisms and require precise spatial and temporal regulation. Interestingly, the migratory cell itself undergoes change during its migration, so temporal development is an important consideration in examining its dynamic cell biology. We are studying the male linker cell, an individual cell in C. elegans that directs the development of the male gonad by carrying out a complex, long-range migration. We have investigated the changes that occur to the linker cell during part of its migration (the third and fourth larval stages) by using genetics and by imaging fluorescent protein-tagged linker cells in live animals. During the mid-third larval stage, we observed the downregulation of one of two C. elegans netrin receptors, unc-5. We show that this downregulation is required for the linker cell to respond differentially to netrin, an extracellular matrix protein present along the ventral bodywall, and results in the linker cell turning from the dorsal to ventral bodywall. During the fourth larval stage, as the linker cell approaches its destination, it changes from a round to arrowhead cell shape with visible membrane extensions. Also during this stage of the migration, the membrane localization of the Rac homology, MIG-2, becomes polarized to the adherent, ventral side of the linker cell. Finally, a zinc metalloprotease, zmp-1, becomes stage-specifically expressed in the linker cell during the fourth larval stage. In an RNAi screen of 508 transcription factors, we identified nhr-67, the ortholog of the Drosophila tailless and vertebrate Tlx genes, to be required for normal linker cell migration. We show that nhr-67/tailless/Tlx functions cell autonomously in the linker cell to execute each of the third and fourth larval stage changes described. We propose that a series of transcriptional regulators, like nhr-67/tailless/Tlx, controls temporal subsets of the linker cell migration program.

Signal Transduction in Development (1186 – 1199)

1186/B344
Pea3 ETS Factors Transduce FGF Signals in Zebrafish Heart Development.
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Fibroblast Growth Factors (FGFs) are secreted molecules that activate the RAS/mitogen-activated protein kinase (MAPK) signaling pathway to ultimately result in gene transcription. In zebrafish development, FGF signaling is required for proper heart formation. To understand how FGFs control this process and dictate gene expression, we are studying the transcription factors that are required to relay FGF activity into a transcriptional response. The FGF target gene, dual specificity phosphatase 6 (dusp6) contains the consensus binding sequences for ETS transcription factors within its promoter. Since several ETS factors are known transcriptional
mediators of MAPK signaling and Pea3 ETS genes (erm, etv5 and pea3) are co-expressed with FGFs, we hypothesized that they are required for FGF target gene expression. However, functional studies in mouse linking Pea3 factors and FGF signaling have been difficult due to redundant roles of these ETS factors in development. The importance of a specific Pea3 binding site within the Dusp6 promoter was determined by EMSAs, and we demonstrated binding of the Etv5 ETS domain to this Pea3 site. In gain-of-function studies, over-expression of an activated form of Etv5 induced dusp6 transcription. In contrast, the concerted depletion of the Pea3 ETS proteins, Erm, Etv5, and Pea3, suppressed expression of known FGF target genes. The knockdown of Pea3, Etv5, and Erm resulted in a decreased number of cardiac progenitors at somitogenesis stages, with the concomitant expansion of the endothelial and hematopoietic progenitors. Thus, Pea3 ETS factors function redundantly to relay FGF signals into a transcriptional read-out during early development to maintain cardiac progenitors. In addition, aberrant cardiac looping was observed in Pea3 ETS depleted embryos suggesting defects in left-right asymmetry. Analysis of Kupffers vesicle revealed that formation of monocilia were severely disrupted, thereby implicating their role in relaying FGF signals necessary for ciliogenesis. During multiple stages of development, the Pea3 ETS factors are required for FGF signaling to regulate several aspects of heart development.

1187/B345
A Membrane-Associated Thioredoxin Required for Plant Growth Migrates from Cell-To-Cell Suggestive of a Role in Intercellular Communication.
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Current evidence indicates that redox state is a critical factor in determining cell fate and regulating cell growth during the development of multicellular organisms. Here we present evidence that thioredoxin (Trx), a small, ubiquitous protein catalyzing thiol-disulfide (SH/S-S) exchange, acts in this capacity in the model plant, Arabidopsis thaliana. Trx is a complex family of regulatory proteins, consisting of six distinct types: f, m, y, x, h and o that reside in different cell compartments and function in an array of processes. The extraplastidic h-type consists of multiple members for which limited functions are known and mutant phenotypes are generally lacking. We have identified a phenotype for a null, loss-of-function mutation in an Arabidopsis Trx h, ATH9. The mutant showed reduced cell size and impaired chloroplast development, resulting in dwarf plants with short roots and small, yellowish leaves. ATH9 was shown to be associated with the plasma membrane using a GFP-tag and to move from cell to cell using an Arabidopsis SCARECROW promoter to drive expression specifically in the single-layer of endodermis. Mutagenesis of ATH9, which has a 17-amino acid N-terminal extension with a highly conserved Gly2 and Cys4, revealed that Gly2 (but not Cys4) is required for membrane binding, possibly due to a myristoylated lipid anchor. Both Gly2 and Cys4 were needed for ATH9 movement. The 3D structure and docking models of ATH9 were consistent with earlier evidence that an ATH9 ortholog from poplar is reduced by glutaredoxin (Grx) and glutathione (GSH), rather than the usual NADP-thioredoxin reductase (NTR). ATH9 thus appears to play a critical role in linking redox status to plant development. The results also extend the known boundaries of Trx in demonstrating that a membrane-bound family member moves and possibly acts in cell-to-cell communication.

1188/B346
Calcium Signaling in Left-Right Development: Clues from the Inversin Mutant Mouse.
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Inversin is a multi-functional, multi-domain protein that is known to play a role in the establishment of the left-right axis. Mice homozygous for an insertion/deletion affecting inversin (inv\(^{ow219/ow219}\)) have consistent situs inversus, cilia at the posterior notochord (PNC) are present and motile, and nodal flow is in the normal leftward direction, albeit slower than in wild-type. The
earliest known left-right asymmetric signal in mouse is an observed elevation of calcium in the crown cells on the left margin of the PNC during timepoints corresponding to nodal flow. We have examined the PNC in inv mutant mice for the pattern of calcium levels to evaluate the relationship between nodal flow, calcium signal and anatomic situs. Live-imaging of e8.5 embryos from invove210/+ intercrosses was performed after loading with Rhod-2, a calcium-sensitive dye. Embryos are genotyped by PCR after imaging. As expected, the calcium signal was left-dominant in 16/17 wild-type embryos (invove210/+ or inv+/+) with 1/17 showing a bilateral pattern. Surprisingly however, it was bilateral in 4/5 invove210/ove210 embryos instead of the expected right-sided calcium signal, which was seen in only 1/5 invove210/ove210 embryos. In addition, as previously reported, we find that the node width is reduced to 49.6 μM in invove210/ove210 mutants as compared to 75.1 μM in wild-type littermates. Expression of asymmetrically expressed genes including lefty2, pitx2 and nodal is being examined in these mice to see if the pattern is bilateral i.e. corresponding to the calcium signal or right-sided, i.e. in accordance with the final anatomic asymmetry. We find that lefty2 is expressed only on the right in these mutants and not in a bilateral pattern, and evaluation of other asymmetric markers is in progress. Together, these data lead to the conclusion that the relationship between nodal flow, asymmetric calcium signal, asymmetric gene expression and anatomic situs is not linear as has been previously hypothesized.

1189/B347
The Novel Src Substrate Timeless Is Required for Controlled Apoptosis during Embryoid Body Cavitation, a Model for Early Mammalian Embryonic Development.
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The Src family of non-receptor protein-tyrosine kinases has been implicated in murine embryonic stem (ES) cell growth, self-renewal, and differentiation. Previous work from our group has shown that suppression of overall Src-family kinase (SFK) activity with the broad-spectrum inhibitor A-419259 blocks ES cell differentiation in response to withdrawal of the essential pluripotency cytokine LIF. In order to identify signaling partners for SFKs in ES cells, the SH3 domain of c-Src was used to precipitate possible target proteins from both pluripotent ES cells and differentiated embryoid bodies (EBs). Several proteins specifically associated with the Src SH3 domain, and were subsequently identified by mass spectrometry as Dynamin, heterogeneous nuclear Ribonucleoprotein K (hnRNPK), and Timeless. Both Dynamin and hnRNPK have been previously identified as Src SH3-interacting proteins, so we focused our attention on Timeless. The Drosophila homolog of Timeless is involved in circadian rhythm, while mammalian Timeless is also essential for embryonic development and has been implicated in DNA damage checkpoint control. To investigate to role of Timeless in embryonic development, Timeless shRNA knockdown mES cell lines were established. Interestingly these lines appeared to be resistant to apoptosis, as staurosporine treatment failed to enhance caspase activity. When these lines were allowed to differentiate to embryoid bodies (EBs), they failed to cavitate. EB cavitation mimics the first known round of developmental apoptosis, resulting in the formation of the embryonic proamniotic cavity. Immunostaining of these Tim-deficient EBs for the self-renewal marker Oct4 showed that the cells present in the failed cavity are pluripotent. Our results identify Timeless as a putative Src substrate in ES cells, where it may function in the earliest stages of development via controlled induction of apoptosis of pluripotent cells, as required for proamniotic cavity formation.

1190/B348
An HMGB1/RAGE/P38 Mapk/Myogenin Axis Modulates Pax7 Expression in Myoblasts by Both Transcriptional and Post-Transcriptional Mechanisms.
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the adult muscle stem cells, and quiescent primary myoblasts do not express RAGE; however, RAGE is rapidly expressed in activated myoblasts and, to a larger extent, in differentiating myoblasts, and its expression is directly related to the expression of myogenin, a muscle-specific transcription factor essential for myogenic differentiation, and inversely related to the expression of Pax7, a transcription factor which is required for quiescence and proliferation of myoblasts/SCs and has to be downregulated for myoblasts/SCs to differentiate. We found that either the blockade of RAGE activity or inactivation of p38 MAPK resulted in downregulation of myogenin and upregulation of Pax7 in myoblasts in differentiation medium (DM), suggesting that RAGE might participate in repression of Pax7 expression via p38 MAPK-dependent induction of myogenin expression. The murine Pax7 promoter contains six myogenin recognition sites, and by chromatin immunoprecipitation assay myogenin bound to three of them in growth medium (GM) and to all of them in DM. Transfection of myoblasts with myogenin caused inhibition of Pax7 (mRNA and protein) expression in GM and DM, while knockdown of myogenin by RNAi resulted in upregulation of Pax7 (mRNA and protein) in DM. Thus, the HMGB1/RAGE/p38 MAPK/myogenin axis might modulate Pax7 transcription. However, blocking the proteasome with MG132 at 6h by the switch of myoblasts to DM (when the myogenin and Pax7 abundances were relatively high and the two proteins were co-expressed in myoblasts) resulted in accumulation of Pax7. at this same time point Pax7 co-immunoprecipitated with myogenin pointing to myogenin-Pax7 interaction. These results suggest that the HMGB1/RAGE/p38 MAPK/myogenin axis modulates Pax7 abundance in differentiating myoblasts via both transcriptional and post-transcriptional mechanisms.

1191/B349
Function and Regulation of MicroRNA-125b in Myogenesis.
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MicroRNAs have recently emerged as key regulators in a wide range of cellular and developmental processes, including skeletal myogenesis. Our microRNA profiling in differentiating C2C12 myocytes revealed potentially novel myogenic microRNAs, among which miR-125b was found to be down-regulated during differentiation. Overexpression of miR-125b suppressed differentiation, whereas inhibition of miR-125b by an LNA-oligo enhanced differentiation in C2C12 cells, suggesting that miR-125b is a negative regulator of myogenesis. Moreover, miR-125b levels decreased during skeletal muscle regeneration in mice, consistent with a negative role of miR-125b in myogenesis in vivo. To identify the target of miR-125b in myogenesis, we considered insulin-like growth factor-2 (Igf2), the 3' UTR of which contained a putative miR-125b target site (prediction by Miranda). Using a reporter system, we confirmed miR-125b targeting of the Igf2 3'UTR with the following evidence: in 293 cells, the Igf2-3'UTR reporter activity was inhibited by miR-125b, and mutation of the putative seed region abolished this regulation; during C2C12 differentiation, the reporter activity increased, which was inhibited by miR-125b overexpression. Importantly, recombinant IGF-II reversed the negative effect of miR-125b overexpression on differentiation, suggesting that IGF-II indeed lies downstream of miR-125b. We further discovered that the down-regulation of miR125b during differentiation was suppressed by rapamycin, a specific inhibitor of mTOR. As expected, rapamycin also inhibited the Igf2-3'UTR reporter activity. The rapamycin effect on miR-125b was reversed by stable expression of a rapamycin-resistant mTOR mutant in C2C12 cells, validating specific targeting of mTOR by rapamycin. Furthermore, stable expression of a rapamycin-resistant/kinase-inactive double mutant of mTOR also protected miR-125b from the effect of rapamycin, suggesting that mTOR signaling regulates miR-125b expression and, subsequently, IGF-II expression, through a kinase-independent mechanism. In summary, we have identified miR-125b as a negative regulator of myogenic differentiation through targeting Igf2, under the control of mTOR signaling.

1192/B350
Studies on the Function of the Misshapen Subfamily of Germinal Center Kinases in Early Xenopus Development.
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TNIK (Traf2 and Nck-interacting kinase) and MINK (Misshapen/NIKs-related kinase) belong to the Misshapen subfamily of GCK family kinases that also includes NIK (Nck-interacting kinase - the third kinase of this subfamily in mammals), MIG-15 (C. elegans) and Misshapen (Drosophila). All the Misshapen subfamily kinases can activate the JNK pathway, functioning as MAP4Ks. For example, in Drosophila dorsal closure Msn signals through the JNK (Basket) cascade, and in determining Planar Cell Polarity (PCP) in epithelial cells it transduces the signal from Frizzled/Disheveled to JNK and p38, coupling a branch of the Wnt signaling pathway with these MAPKs. In Xenopus laevis the function of the Misshapen subfamily GCKs has not been studied. We obtained the full length cDNA of xMINK and xTNIK. Knock-down of either xTNIK or xMINK with antisense Morpholinos gives similar phenotypes, it disrupts the process of convergence and extension that leads to neural tube closure defects and shortened antero-posterior axis. Overexpression of dominant negative forms of xTNIK and xMINK lacking the kinase domain also causes a neural tube closure defect and can be rescued by coexpression of either xTNIK or xMINK lacking C-terminal homology domain. Embryos with disrupted Dishevelled signaling display similar phenotypes to knock-down xMINK and xTNIK embryos. Both xTNIK and xMINK colocalize with Dishevelled in the same cytoplasmic speckles and are transported to the membrane together with Dishevelled upon the coexpression of the Wnt receptor Frizzled. The data present the first demonstration that the Msn family of kinases interact physically and are cotransported with Dishevelled and very probably function as a TNIK-MINK heterodimer.

1193/B351
Glypican Core Proteins Potentiate Cell-Autonomous Hedgehog Response.
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The distribution and activities of morphogenic signaling proteins such as Hedgehog (Hh) and Wingless (Wg) depend on heparan sulfate proteoglycans (HSPGs). HSPGs consist of a core protein with covalently attached heparan sulfate glycosaminoglycan (GAG) chains. We report that the unmodified core protein of Dally-like (Dlp), an HSPG required for cell autonomous Hh response in Drosophila embryos, alone suffices to rescue embryonic Hh signaling defects. Using a Drosophila clone-8 (cl-8) cultured cell assay to focus on Hh response, we tested the structural requirements of Dlp for potentiation of the Hh signal. Membrane tethering, but not specifically the glycosylphosphatidylinositol (GPI) linkage characteristic of glypicans, is critical for cell autonomous activity. By testing a series of chimeric fusions to the divergent glypican Dally, we isolated a minimal core sequence sufficient to rescue Hh response. We extended our analysis to mammalian glycans and found that mouse GPC4 and GPC6 were capable of rescuing dlp RNAi when expressed in cl-8 cells. As with Dlp, the potentiation of Hh response by mammalian glycans did not require GAG chain modifications, indicating that conserved structural features of the core protein might interact with components of the Hh pathway. Conversely, Dally, GPC2, GPC3, and GPC5, exhibited trans-dominant negative activity, suggesting the divergence of the two Drosophila and six mammalian glycans into two functional families: an activating family that rescues cell autonomous Dlp function in Hh response and a family that inhibits Hh response. Thus, in addition to the previously established requirement for HSPG GAG chains in Hh movement, these findings demonstrate for the first time a positive, cell autonomous role for a glypican core protein in morphogen response in vivo, and suggest the conservation of a network of antagonistic glypican activities in regulation of Hh response.
Cell signaling takes place in the three-dimensional context of tissues, but for many major signaling pathways, the subcellular architecture of the signaling apparatus is not well understood. The Hedgehog (Hh) signaling pathway is a vitally important but incompletely understood mediator of both normal development and human diseases. Loss of function of certain Hh modulators can have very different effects on Hh signaling in cell lines versus tissues, suggesting that cell-cell interactions and/or cell morphology can significantly affect Hh signaling. Using Drosophila as a model system, we have identified known and novel Hh regulators that affect the subcellular localization of Hh transducers, as well as tissue morphology and organelar localization. Our objective is to characterize how these Hh regulators affect cellular morphology and how this, in turn, affects development, using a combination of genetics and cell biology. As a first step, we have identified the first phosphatase involved in Hh signaling, a specific isotype of the PP2A phosphatase, and shown that overexpression of the PP2A regulatory subunit, Widerborst (Wdb) causes Hh dependent outgrowth perpendicular to the plane of the wing imaginal disc. Overexpression of an N-terminally truncated form of Wdb, previously demonstrated to inhibit planar cell polarity, causes robust growth perpendicular to the plane of the disc. We have also shown that one of the kinases that may be counteracted by PP2A, the PKA-C1 kinase, causes changes in the wing disc cellular architecture when overexpressed, apparently by causing changes in apicobasal shape. We have also identified the first deacetylase implicated in Hh signaling, the HDAC3 deacetylase. HDAC3 in Drosophila is localized principally at the apicobasal domains of wing disc cells and seems to accumulate in regions of increased microtubule density. We conclude that the subcellular localization of Hh regulators is important for their function in the pathway, and modification of this localization can lead to profound changes in cell and tissue morphology in developing animals.

Wnt-Frizzled signaling appears to play significant role during retinal development. Previously, we reported an essential role of Frizzled5 (Fz5) in retinal development (Liu C et al., 2008). We hypothesize that multiple Frizzled receptors orchestrate Wnt-signaling as a redundant system. To address this, we have focused on another receptor, Frizzled 8(Fz8), which shares high similarity in the extracellular cystein rich domain (CRD) with Fz5, and is also activated by the same Wnt ligand, Wnt9b, at least in vitro. A knock-in beta-gal reporter was used to study Fz8 expression pattern. In adult retina, beta-gal reporter activity showed in all three neuronal layers, with a stronger expression in photoreceptors inner segment. Expression of Fz8 in the inner nuclear and ganglion cell layer co-localized to Islet-1 in a subset of amacrine cells. In addition, some of Frizzled 8 mutants showed abnormal electroretinogram (ERG), which likely results from abnormalities of photoreceptors. We are generating Fz5cko-/-;Fz8-/- compound knockout mutants and are evaluating roles of Frizzled receptors in different retinal cell types.

The Hedgehog (Hh) pathway has critical roles in patterning and growth in a variety of organisms ranging from insects to vertebrates. Dysregulation of the Hh pathway in humans is associated with a variety of congenital anomalies and various cancer types. Hh encodes a secreted polypeptide which undergoes a unique and conserved processing mechanism required for
generation and release of an active molecule. The final, fully active product, HhNp, is both cholesterol modified at its C-terminus and palmitoylated at its N-terminus. A critical question relates to how the Hh ligand, a dually lipidated secreted protein, is transported from the producing cells to the responsive cells and elicits different responses in target fields. Using cell culture based assays we are investigating release of the Hh ligand from the producing cells as well as analyzing mechanistically the role of various factors whose involvement has been defined genetically. We found that Shifted, the Drosophila homologue of human Wnt Inhibitory Factor-1, has an important role in Hh pathway by increasing the potency of HhNp conditioned media and the levels of soluble HhNp. We established that Shifted can interact and enhance the activity of HhN, a construct truncated after G257 at the site of processing, which is not autoprocessed and consequently not cholesterol modified. In zebrafish, Scube2 (Signal sequence, Cub domain, EGF related) was described by genetic studies as a positive component of Shh pathway which can modulate the pathway activity non cell-autonomously. Using a mammalian cell culture assay, we found that members of the Scube protein family can enhance the potency and levels of soluble ShhNp by directly binding to it. We found that Scube EGF domains are not required for Scube activity in the Shh pathway. Using both Drosophila and mammalian cell culture based assays, we can address the mechanism by which the levels and potencies of HhNp/ShhNp can be modulated by secreted factors described genetically.

1197/B355
Mediator/Trap230 and Ethanol May Influence Zebrafish Neural Crest Cell Development and Differentiation through the Wnt Beta-Catenin Signaling Pathway.
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Exposure to ethanol during embryogenesis results in the disruption of multiple developmental pathways, leading to a wide range of developmental defects. In order to investigate potential mechanisms by which ethanol exposure affects neural crest cell development, we have employed genetic and pharmacologic analyses of pigment pattern formation in zebrafish. In homozygous kohtalo (kto82) mutant zebrafish, which have a point mutation in the mediator complex gene, med12 (trap230), embryos are hypopigmented and lack iridophores. Treating wildtype and mutant zebrafish embryos with various concentrations of ethanol throughout early development allowed us to observe changes in pigment cell fates and lineages. Exposing mid-blastula stage embryos to ethanol for a 24-hour period resulted in iridophore reduction and increases in melanophores and xanthophores, suggesting a cell fate shift. Although kto embryos lack iridophores, ethanol treated mutants also showed this increase in melanophores and xanthophores. Increasing these ethanol concentrations resulted in a greater stem cell shift towards melanogenesis rather than the xanthophore lineage. Chromatophore migration, general patterning and development remained unaffected. Embryos treated 24-hours post fertilization showed a slight decrease of chromatophores, highlighting that ethanol induced changes in cell differentiation depend on the time of treatment. Taken together these results suggest that ethanol interferes with critical signaling transduction pathways, primarily the Wnt Beta-catenin pathway, during specific developmental periods, to cause a shift towards melanoblasts and xanthoblasts.

1198/B356
GATA6 Induces Wnt6 Expression during Primitive Endoderm Differentiation.
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Mouse F9 teratocarcinoma cells differentiate in culture into primitive endoderm when treated with retinoic acid (RA) and into parietal endoderm with subsequent treatment with dibutyryl cAMP. This model has been studied extensively as these chemically-induced events mimic one of the earliest epithelial-to-mesenchymal transitions in mouse embryogenesis. During primitive endoderm differentiation, Wnt6 expression is up-regulated by RA, leading to the activation of the
canonical Wnt/β-catenin signaling pathway. The factors responsible for activating Wnt6 are not known, but in silico analysis reveals that its promoter region contains a putative binding site for the transcription factor GATA6. The objective of our research was to test whether or not ectopic expression of Gata6 induces Wnt6, and if this was sufficient to induce F9 cells into primitive and/or parietal endoderm. In this study, Gata6 expression was found to be up-regulated during primitive and parietal endoderm differentiation. Ectopic expression of Gata6 alone induced biochemical, molecular, and morphological markers of primitive endoderm and was sufficient in up-regulating Wnt6 expression. Furthermore, this up-regulation was accompanied by the activation of the canonical Wnt/β-catenin signaling pathway, as evident by the increase in phospho-GSK3β levels. Also, when treated with dibutyryl cAMP, Gata6 expressing cells completed the epithelial-to-mesenchymal transition and differentiated into parietal endoderm. Together, these results show that ectopic expression of Gata6, working to induce Wnt6, is sufficient to activate the canonical β-catenin signaling pathway required for the specification of primitive endoderm.

1199/B357
Shared Molecular Mechanisms Regulate Multiple Catenin Proteins.
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The Wnt pathways contribute to many processes in cell and developmental biology. Recent work has shown that p120-catenin binds and is stabilized by Frodo, a protein earlier found to interact with Dishevelled. P120-catenin is a member of the p120 sub-family of catenins (p120-subF), and as β-catenin, it has a central Armadillo domain capable of binding cadherin cytoplasmic tails. In the case of p120-subF members, cadherin association leads to increased cadherin stability. Distinct from β-catenin, p120-subF members additionally modulate small Rho-family GTPases. Further, in the nucleus, p120-catenin itself relieves repression conferred by the novel POZ/zinc-finger transcription factor Kaiso. In the case of the Xenopus Siamois gene promoter, which contains both Kaiso and TCF binding sites, our published work suggests that the p120-catenin/Kaiso pathway acts in parallel with the established β-catenin/TCF (canonical Wnt) pathway. In the present work, employing Xenopus embryos and mammalian cell lines, we report that the degradation machinery of the canonical Wnt pathway modulates p120-catenin protein stability through mechanisms shared with β-catenin. Exogenous expression of destruction-complex components such as GSK3β or Axin promotes p120-catenin degradation, and consequently, is able to rescue developmental phenotypes resulting from p120 over-expression. Likewise, Axin or GSK3β depletion coordinately increases p120-catenin and β-catenin levels in vivo. At the primary sequence level, we resolved conserved GSK3β phosphorylation sites in the amino-terminal region of p120-catenin. Point-mutagenesis of these residues inhibited the association of destruction complex proteins, resulting in p120-catenin stabilization. As would be predicted, these p120 mutants exhibited increased signaling activity in the context of the p120/Kaiso pathway. Importantly, we found that two additional p120-catenin family members, ARVCF-catenin and delta-catenin, in common with β-catenin and p120, associate with Axin, and are degraded in its presence. Based upon our embryo and cell line findings, canonical Wnt signals are likely to have a broad impact upon catenin biology in vertebrate development and perhaps in cancer progression.

Stem Cells II (1200 – 1224)

1200/B358
Dental Pulp Stem Cell Differentiation Is Affected by the Mechanical Properties of Polybutadiene Substrates.
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Adult mesenchymal stem cells have the potential to differentiate into various cell lineages when induced by specific chemical inducers. It was shown that brief chemical induction followed by interaction of stem cells with matrices of variable elasticity could influence lineage specification of adult stem cells (Engler et al, 2006). In this study Dental Pulp Stem Cells (DPSCs), known to differentiate into a range of cell lineages, were used to assess the impact of surface mechanics on biomineralization. Polybutadiene (PB) was used as surface to provide the mechanical stimulus but without the initial brief chemical induction. Atomic Force Microscopy (AFM) on Shear Modulation Force Microscopy (SMFM) mode indicated that the spun-cast PB films substrate stiffness corresponds to the thicknesses of films. Spun-cast polybutadiene (PB) films of 200 nm and 2000 nm thickness were used to generate surfaces with a 4-fold range of stiffness. DPSCs were plated on these substrates and cultured in media in the presence or absence of dexamethasone; on tissue culture plastic dexamethasone is a requirement for biomineralization by DPSC. After 28 days incubation, Laser Scanning Confocal Microscopy (LSCM) and Hg florescence spectroscopy revealed that crystals were present only on the stiffer PB samples. Further analyses using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray analysis (EDX analysis) indicated that the crystals were composed of calcium phosphate. The Glancing Incidence Diffraction (GID) spectra obtained from these deposits were similar to those obtained from hydroxyapatite standards. Taken together the positive correlation between substrate mechanics, cell stiffness, calcium phosphate deposition and osteocalcin expression supports the notion that DPSC differentiation and biomineralization can be regulated by substrate mechanics. Use of biocompatible materials, like PB, in building scaffolds with variable mechanical properties could lead to lineage specification without the need of chemical inducers and possible safer use in tissue engineering and implantation. Engler,AJ; Sen,S; Sweeney, HL and Dennis E. Discher, DE (2006) Matrix Elasticity Directs Stem Cell Lineage Specification. Cell 126, 677-689.

1201/B359
CD44 Expression by Umbilical Cord Stem Cells Increases after Compressive Stimulation in a 3D Gel Environment.
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Stem cell-based regenerative medicine may be a potential approach for articular cartilage repair. Stem cells are attractive candidates for therapeutics because of their plasticity, which may be affected by both biochemical and biophysical stimuli. Yet, their current utility is diminished by our limited grasp of how signals direct cell fate toward chondrogenesis. Here we investigate the effect of compressive mechanical stimulation on novel umbilical cord stem cells (UCSC) seeded in 3D gel scaffolds. Expanded UCSC isolated from male human umbilical cords were photoencapsulated in 3D hydrogels. Gel constructs were made from chemically modified hyaluronic acid macromonomers, dissolved in UCSC-cell suspensions, which were subsequently crosslinked via UV exposure to form 3D gels of 3.0 - 4.3x10⁶ cells/gel. Following 48 hours of compressive loading (13.5% strain, 1Hz) with an oscillatory compression device, the gels were enzymatically digested to collect the cells for analysis. Under monolayer control conditions, this novel population of UCSCs exhibits a stable mesenchymal stem cell (MSC)-like phenotype, having near 100% positivity for mesenchymal surface markers CD44, CD73, CD90, and CD105. After 48 hours in static 3D constructs, we observed a slight decrease in expression of CD44, CD73, and CD90 and a dramatic drop in CD105 and CD146. After dynamic compression, we observe significant decreases in levels of CD73, CD90, CD105, and CD146 as compared to both pre-stimulated controls and static controls. However, CD44 expression significantly increased in
compression-stimulated constructs as compared to static constructs. After compressive loading, the UC cells appear to become more chondrocyte-like, reducing their MSC-like surface marker expression while upregulating the HA ligand CD44. CD44 has been shown to be important for cartilage matrix assembly and retention. Pre-stimulation of stem and progenitors may be necessary to control stem cell fate in stem cell-based therapy for articular cartilage repair. In sum, this demonstrates that UC MSC-like cells respond to these stimuli, illustrating the importance of examining cell behavior under dynamic loading and in 3D environments, which mimic the physiological stresses.

1202/B360
Maintaining Pluripotency of Human Embryonic Stem Cells with Nanotopography.
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To realize the potential of human embryonic stem (hES) cell based regenerative therapy, self-renewal and expansion of hES cells in culture is a challenge that must be addressed. This challenge can be met by utilizing synthetic culture substrates whereby providing feeder-, xeno-, and possibly cytokine-free hES cell expansion cultures. We have developed substrates with nanotopographical cues (NTCs) and have evidence that these NTCs promote self-renewal of hES cells in the absence of bFGF supplementation. The NTCs are fabricated from polystyrene using nanoimprint lithography and consist of various types; 50 ± 5 nm diameter nanopillars (NP) arranged in an approximately hexagonal closed packed (HCP) array of 50 to 300 nm spacing, and HCP nanoholes (NH) with diameters that vary from 100 to 400 nm. Human fibronectin (FN) and mouse Matrigel (MG) are used to coat the NTCs with a thin 10-20 nm layer for cell adhesion. hES cells are dissociated into single cells from hES colonies co-cultured with MEF feeders and plated onto the NTCs at a density of 2000 cells/cm^2. at this density, any influence from the low number of feeders passaged onto the NTCs is negligible and the hES cell fate is dominated by the cell-substrate interaction. at day 8, the percentage of pluripotent cells are quantified by immunostaining the cells with fluorescent Oct4 antibodies. on MG coated NTCs, less than half of the cells remain pluripotent. on FN coated NTCs a higher proportion of cells remain pluripotent. A trend of increasing number of undifferentiated cells is found for FN-NPs with increased spacing resulting in 300 nm spaced FN-NPs having a significantly higher percentage of Oct4+ cells compared to flat substrates (91% vs. 77%, t-test, p=0.025). In addition, hES cells cultured on FN-NH NTCs result in a higher percentage of undifferentiated cells compared to FN-NP NTCs (97% undifferentiated cells for 200 nm NH, t-test, p=0.013). Our results provide evidence that the cell-nanoenvironment interaction can determine hESC fate. The tantalizing possibility of xeno-free, feeder-free, and cytokine-free hESC expansion with synthetic culture substrates is hinted, and further work needs to be done to elucidate the signaling and pathways involved.

1203/B361
The Response of Mouse Embryonic Stem Cells to Optical Forces: The Role of Mechanics in Pluripotency and Embryogenesis.
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We set out to discover the role of the mechanical properties of mouse embryonic stem cells (ESCs) in their pluripotency. Pluripotency of ESCs is highly correlated to the expression of nanog, a transcription factor found only in ESCs that is critical in the self-renewal of undifferentiated stem cells. To test the mechanical properties of ESCs, we investigated the response of ESCs to optical forces using the optical stretcher, a dual-beam optical trap that uses radiation pressure to hold and manipulate dielectric objects such as biological cells. We FACS sorted ESCs into high nanog expressing (HN) and low nanog expressing (LN) groups, and discovered vital connections
between the mechanical properties of ESCs and their pluripotency via expression of nanog. HN cells are more plastic while LN cells are more elastic, indicating differences in the ways in which ESCs respond to forces in their environment as a function of their nanog expression, and therefore their pluripotency. In addition to presenting results of control studies which further indicate a correlation between nanog expression and mechanical response, we will discuss possible mechanisms underlying these differences, including an exploration of the role of cytoskeletal components such as the actomyosin network and microtubules. We are currently establishing an In Vivo connection to this work by sorting the mouse blastocyst into cells from the inner cell mass and trophectoderm and testing the differences in their mechanical properties using the optical stretcher. We will present the highly intriguing results of this study which will elucidate the differences between the responses of inner cell mass cells and trophoblasts to forces in their environment, and relate these results to the results of the cultured ESC study. Finally, we will attempt to clarify the role of cell mechanics in pluripotency and embryogenesis.

1204/B362
Selective Accumulation of Post-Division Midbodies in Stem Cells.
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The midbody (MB) is a large, singular, nonmembranous organelle formed late in mitosis between the two nascent daughter cells and plays a crucial role in the final stage of cell division, abscission. The composition of the MB is complex, being comprised of over 500 proteins. These include the putative stem cell marker prominin-1/CD133 and the cell fate determinant Cdc2, molecules that suggest functions of the MB beyond abscission. Moreover, recent work from our lab and others demonstrated that post-mitotic MBs, or midbody derivatives (MBds), accumulate in cells. To understand the nature of cells that accumulate MBds, we performed immunohistochemical analysis of tissues and found that MBds accumulated in stem cells in several niches including the testes, hair follicle, and gut. Immunofluorescence analysis of cells derived from diverse tissues also revealed multiple MBds in stem cells and tumor cells. Remarkably, MBds were not found in normal proliferating non-cancer, non-stem cells (e.g. primary MEFs) in tissues or cell lines. Moreover, MBds were reduced upon differentiation of human embryonic stem cells, and increased upon induction of pluripotency in mature fibroblasts. To address the mechanism of selective MBd accumulation we modulated protein degradation pathways. In normal proliferating cells, MBds were found primarily in lysosomes and they accumulated when lysosomal proteolysis or autophagy was blocked. Proteasome-mediated degradation did not appear to be involved in MBd elimination. In tumor and stem cells, MBds were primarily free in the cytoplasm and did not accumulate upon lysosomal inhibition. Nevertheless, they were decreased when autophagy was activated or were further increased when the autophagic receptors were depleted. These results demonstrate that autophagy plays a major role in the retention or loss of MBds and this is different in different cell types. More specifically, the high autophagic flux in normal cells favors MBd degradation, while low autophagic flux in stem cells and most tumor cells studied, favors MBd accumulation. Based on our data and the presence of cell fate determinants on MBs, we propose that MBds may contribute to the function of stem cells and putative cancer stem cells.

1205/B363
Epigenetic Actions of Insulin-Like Growth Factors (IGFs) Can Selectively Target the Oct4 Promoter Changing the Fate of Mesenchymal Stem Cells (MSCs).
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Cell growth and differentiation are controlled through activation or silencing of genes in a temporal and spatial manner. Micro-environmental changes lead to epigenetic events initiated by the polycomb group proteins (PcG) such as EZH2. IGFs, as an important component of the stem
cell niche, are known to regulate cell differentiation, proliferation and survival. We have previously demonstrated that IGF-I sustains the osteogenic differentiation of MSCs by increasing Runx2 expression and decreasing OCT4 levels. Thus, our objective was to analyze the role of IGFs in the regulation of EZH2 to initiate changes in placental MSC fate. Placental MSCs were treated with IGFs and processed for ChIP assay using an EZH2 antibody. Other markers such as pAKT, pERK1/2, OCT4, RUNX-2, cyclin D1 and histone 3K27 were identified by western blot. Low doses of IGF-I (10-50 ng/mL) significantly increased the levels of pERK1/2 and Cyclin D1, which is known to enhance the EZH2 production. On the other hand, high doses of IGF-I (50-200 ng/mL) increased the levels of pAKT which is recognized to repress both the EZH2 and meH3K27 activity, thus inducing gene repression events. Furthermore, we demonstrated that IGF-I alters the activity of EZH2 at the OCT4 gene promoter, silencing the expression of the OCT4 transcription factor and thus potentially changing the stem cell fate. Polycomb proteins are known to maintain transcriptional repression of the stem cell genes responsible for their renewal and we show that these proteins can be selectively regulated by IGFs, with IGF-I proving to be a better modulator than IGF-II. By multiple rounds of PCR, we mapped the recruitment of EZH2 at the OCT4 promoter and identified at least three regulatory regions dependent on IGF-I treatment, located in the distal (-)2340-2042 and proximal (-)1725-1564 enhancer areas but not in the proximal promoter segment of this gene. We conclude that IGF-I, through activation of signaling proteins such as pERK and pAKT, selectively regulates the actions of the EZH2 protein at the OCT4 promoter, ultimately promoting a change in MSC fate towards cell differentiation.

1206/B364
Activation of mTOR-Mediated Protein Translation Induces Differentiation in Pluripotent Human Embryonic Stem Cells.

Deciding to exit pluripotency and undergo differentiation is of singular importance for pluripotent cells, including embryonic stem cells (ESCs). The molecular mechanisms for these decisions to differentiate, as well as reversing those decisions during induced pluripotency (IPS), have focused largely on transcriptomic controls including Oct4, Nanog, Sox2 and Rex1 among others. Here, we explore the role of translational control for the maintenance of pluripotency and the decisions to differentiate. To explore translational control in human embryonic stem cells (hESC), as well as the responsible regulatory signaling pathways, we investigated both protein translation in pluripotent versus differentiated hESCs and examined the translational regulatory mTORC1 (mammalian target of rapamycin complex 1)/p70 S6K pathway. Global protein translation is significantly reduced in hESCs compared to their differentiated progeny. Furthermore, p70 S6K activation is restricted in hESCs compared to differentiated fibroblast-like cells. Disruption of p70 S6K-mediated translation by rapamycin or siRNA knockdown in undifferentiated hESCs does not alter their pluripotent state, i.e. it does not impair colony formation, cell viability or expression of the pluripotency marker Oct4. However, expression of constitutively active p70 S6K, but not wild-type p70 S6K, induces differentiation as demonstrated by decreased Oct4 expression and changes in cellular morphology. Additionally, hESCs exhibit high levels of the mTORC1/p70 S6K inhibitory complex TSC1/TSC2 and preferentially express more rapamycin insensitive mTORC2 compared to differentiated cells. siRNA-mediated knockdown of both TSC2 and Rictor elevates p70 S6K activation and induces differentiation of hESCs. These results suggest that hESCs tightly regulate mTORC1/p70 S6K-mediated protein translation in order to maintain a pluripotent state as well as implicate a novel role for protein synthesis as a driving force behind hESC differentiation.
Long-Term Feeder-Free Culture of Pluripotent Human ES Cells in a Defined, Xeno-Free, Low-Growth Factor Culture Medium.

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The most widely-used culture systems for the maintenance and expansion of pluripotent stem cells utilize components such as serum or serum-replacers along with feeder layers of mouse embryonic fibroblasts (MEFs). However, the use of these products for pluripotent stem cell culture is not optimal for a number of reasons. First, components such as serum or feeder cells are undefined, and therefore introduce a host of unknown variables into the culture environment. Second, the performance of serum, serum-replacer or feeder cells can vary significantly from batch to batch, requiring additional time and effort to test these reagents prior to experimental use. Finally, fetal bovine serum, most serum-replacement products and MEFs are all of animal origin, and their use for pluripotent stem cell culture precludes the stem cells from being suitable for clinical applications. To address these issues, we have developed a human pluripotent stem cell culture medium that does not require the use of feeder cells, and which contains components that are only synthetic or of human origin (i.e., "xeno-free"). Furthermore, this medium contains low levels of critical growth factors such as basic FGF, in contrast to many other pluripotent stem cell culture media formulations. This xeno-free culture medium has been tested with human ES cells in feeder-free culture for more than 20 passages, and has been shown to maintain the pluripotent state of these cells by the following criteria: 1) pluripotent marker expression (OCT4, SSEA-4, TRA-1-81) of more than 95% of the cells as analyzed by immunocytochemistry and flow cytometry; 2) maintenance of a normal karyotype; and 3) the ability to differentiate to all three germ layers as examined by both In Vitro (embryoid body) and In Vivo (teratoma) methods. Cells cultured in this medium showed expected morphology, being present in tightly packed colonies with well-defined boundaries. Finally, human ES cells attached with very high efficiency to the substrate after being passaged in this medium, resulting in more rapid expansion due to decreased loss of cells.

Identification of Smad2 Target Genes Involved in Definitive Endoderm Formation.

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It is well-established that Nodal signaling is one of the key pathways governing vertebrate definitive endoderm (DE) formation. Extracellular Nodal is transduced intracellularly by the Smad2 and/or Smad3 effector proteins, which in cooperation with nuclear partners such as FoxH1, activate key genes during DE formation. Despite its identification nearly fifteen years ago, only a handful of direct transcriptional targets of Nodal have been identified. These include Pitx2, Goosecoid, Lefty and Nodal itself. Here, we aim to comprehensively identify Nodal downstream targets by mimicking DE formation in-vitro with mouse embryonic stem cells (mESCs). Smad2-deficient mESCs fail to incorporate into the DE lineage in chimeric mouse embryos (Tremblay et al., 2000 Development). Similarly, specific loss of Smad2 within the epiblast yields mutant embryos lacking DE (Vincent et al., 2005 Genes & Dev). We were able to translate these In Vivo findings to an In Vitro directed differentiation model, whereby Smad2-deficient mESCs in contrast to wild type mESCs, failed to form DE when exposed to recently described efficient protocol for DE formation (Morrison et al., 2008 Cell Stem Cell). This differentiation strategy relies on recombinant Activin, a Nodal mimic, to stimulate differentiation. With this In Vitro platform, we have thus screened for putative Smad2 targets using Illumina® MouseRef-8 v2 Expression Bead Chip. We have since obtained snapshots of differential gene expression profiles between Smad2 deficient ES cells and wild type ES cells at early, mid and late time points during DE differentiation. Several differentially expressed candidates, encoding diverse proteins ranging...
from transcription factors to signaling molecules to enzymes, have been shortlisted from this screen. We are currently functionalizing these candidates both in-vitro and In Vivo (using mouse and Xenopus embryo model) in order to establish their place, if deserved, as rightful Nodal targets.

1209/B367
Search of STAT3 Target Genes and the Functions in the mES Cells.
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The LIF/STAT3 signaling pathway has been suggested to play an essential role in the maintenance of self-renewal and pluripotency in mES cells. Mouse embryonic stem (mES) cells can proliferate indefinitely in an undifferentiated state in the presence of a cytokine, leukemia inhibitory factor (LIF). We performed DD-RT PCR (differential display-reverse transcription PCR) in the presence or absence of LIF. We finally selected 8 genes whose expressions were significantly dependent upon the presence of LIF. Among them, Jmjd1a (jumonji domain containing 1a) was down-regulated after LIF withdrawal, and was subjected to further investigation. We also look for the STAT3-regulated genes in mES cells, X-ChIP (chromatin cross-linking and immunoprecipitation) was employed with STAT3-specific antibody. Using We isolated several candidates, such as ZFP-57, FoxJ1, and so on. FoxJ1, one of the X-ChIP candidates showing increased expression after LIF withdrawal, is a member of forkhead box family of transcription factors. It was also found using EMSA analysis that STAT3 could bind to the promoter region of FoxJ1 and Jmjd1a. These suggest that FoxJ1 and Jmjd1a are regulated by the LIF/STAT3 signaling pathway. In order to study the function of these candidate genes, we established a Tet-on gene induction system; target gene expression is turned on by the addition of tetracycline or doxycycline to the culture media, in mES cell. Using this mES cell Tet-on system, we are trying to elucidate the functions of the Jmjd1a and Foxj1. With further study, we are hoping to understand the mechanisms through which STAT3 regulates the differentiation and self-renewal of mES cells.

1210/B368
Cdk2ap2, a Novel Regulator of Self-Renewal of Murine Embryonic Stem Cells.
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Embryonic stem cells are being increasingly looked at as the 'holy grail' for replacement therapeutics for various diseases but adverse effects resulting from unregulated growth and differentiation are hampering advances in the field. Although a substantial amount of information has been generated for understanding the signals involved in early development, survival mechanisms involved in early embryogenesis remain poorly understood. This study was designed to identify the function of Cdk2ap2, a negative regulator of Cdk2 function. We have generated murine embryonic stem cell lines (mESC) with homozygous disruption of the Cdk2ap2 locus (Cdk2ap2-/-). Loss of Cdk2ap2 expression resulted in spontaneous differentiation of mESC when grown under self-renewing conditions. RT-PCR analyses for expression of the Cdk2ap2-/- mESC showed a reduction in the expression of Nanog, a marker of stem cell pluripotency, and increased expression of the various markers of lineage differentiation, including Brachyury (mesoderm) and Afp (endoderm), supporting the spontaneous differentiation phenotype in these cells. In a teratoma assay to analyze the differentiation potential of the mESC Cdk2ap2-/- cells failed to form tumors. In addition, Cdk2ap2-/- mESC failed to form 'proper' embryoid bodies In Vitro and showed an increased apoptosis following induction of differentiation, either by withdrawal of Leukemia Inhibitory Factor (LIF) or by treatment with Retinoic Acid (RA). Consistent with a role for the Cdk2ap family in negatively regulating cell cycle, we saw a reduction in the levels of the Retinoblastoma gene (pRb) and an increase in expression of E2F regulated genes. This suggested that loss of Cdk2ap2 expression resulted in deregulation of E2F transcriptional activity and an increase in the pro-apoptotic function of E2F1. These findings support a novel
function for Cdk2ap2 in regulation of self-renewal of mESC and cell survival during differentiation. Thus, Cdk2ap2 represents a potential missing link connecting regulation of cell cycle, differentiation, and survival during early embryonic development. Furthermore, our data suggests that Cdk2ap2 can be a potential target for addressing the current problems in stem cell therapeutics.

1211/B369
Gene Expression Profiling of Five Mouse ES Cell Lines during Stem Cell Differentiation.
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Gene expression analysis of ES cell differentiation In Vitro presents multiple challenges due to limited numbers of cells and the inherent heterogeneity of embryoid bodies. A rapid and robust sample prep methodology coupled with TaqMan® and SYBR® based expression analysis of signature genes for ES cells and differentiated cell types was established for investigating molecular and cellular changes during ES cell differentiation. The Applied Biosystems TaqMan® Cells-to-CT™ Kit was used in conjunction with the TaqMan® Mouse Stem Cell Pluripotency Arrays to determine the relative expression of 96 genes in five genetically distinct embryonic stem cell lines (derived from C57BL6/N, FVB/N, DBA/2N, C3H/HeN, and Balb/cN mouse strains) at day 0, day 4, day 8 and day 11 of differentiation. The ES lines were derived at Predictive Biology. ES cells were plated in ES medium without LIF for production of embryoid bodies (EBs). Lysates were produced using Cells-to-CT™ Lysis Solution, reverse transcribed, added to TaqMan® Gene Expression Master Mix, and run on TaqMan® Mouse Stem Cell Pluripotency Arrays. Expression of many pluripotency genes dropped significantly during differentiation, while expression of some ES cell marker genes showed little change during 11 days of In Vitro differentiation. Some genes exhibited highly similar expression in the five ES cell lines, while others varied significantly in different genetic backgrounds. Ten representative genes from the Pluripotency Array were selected based on differential expression across differentiation stage or genetic background and re-tested individually using the Cells-to-CT™ Power SYBR® kit and the TaqMan® Gene Expression Cells-to-CT™ kit. Confirmation of the initial TaqMan® Array gene expression results demonstrates that Cells-to-CT™ technology used in concert with TaqMan array, individual TaqMan, or SYBR® based qPCR are all viable options for researchers seeking to perform gene expression analysis on ES cells and embryoid bodies.

1212/B370
Reducing Histone 3 Lysine 9 Trimethylation Levels in Embryonic Stem Cells Improves Nuclear Reprogramming into Cloned Mouse Embryos.
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During nuclear transfer (NT) the donor nuclei must be reprogrammed from a lineage-specific to a totipotent state. This has been achieved in numerous species; however, cloning efficiency remains low, mainly due to incorrect epigenetic reprogramming of the donor cell. One epigenetic mark which is known to be difficult to reprogram is the trimethylation of lysine 9 in histone H3 (H3K9Me3). We hypothesized that H3K9Me3 marks are a major limit for successful epigenetic reprogramming. In order to test this idea, we have engineered mouse embryonic stem (ES) cells for the tetracycline-inducible expression of an EGFP fusion protein with 1) the functional H3K9Me3-specific histone demethylase jmd2b (jmd2b-EGFP) or 2) a non-functional mutant (*jmd2b-EGFP). Approximately 94% and 88% of the cells can be induced for the expression of jmd2b-EGFP and *jmd2b-EGFP, respectively. Induction of jmd2-EGFP resulted in ~50% reduction in H3K9Me3 immunofluorescence signal compared to non-induced cells and induced *jmd2b-EGFP cells, respectively. This initial difference in H3K9Me3 levels disappeared within 1 hour following NT, indicating re-acquisition of H3K9Me3 marks in the oocyte cytoplasm. Despite
this rapid reprogramming, the reduction of H3K9Me3 levels prior to NT significantly improved in-vitro development into cloned embryos from induced vs. non-induced jmjd2b-EGFP cells (108/223=48% vs. 89/238=37%, respectively, P<0.05). for induced vs. non-induced *jmjd2b-EGFP control donors, there was no difference in development. Following embryo transfer and recovery around D13.5, no difference in implantation rates between induced vs. non-induced jmjd2b-EGFP-derived embryos was observed (17/54=32% vs.13/28=46%, respectively). This suggests that reduced H3K9Me3 levels improve in-vitro but not in-vivo development. Alternatively, the ES clone used for NT may not be suitable for full-term development. To test this possibility, we have generated one live chimeric offspring from jmjd2b-EGFP cells and are currently evaluating its germ-line contribution.

1213/B371
Reprogramming of Adult Human Fibroblasts by Xenopus Egg Extract Fractions.
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Direct reprogramming of a differentiated somatic cell into a pluripotent cell would offer an alternative to applications in regenerative medicine that currently depend on, either embryonic stem (ES) cells, adult stem cells or induced pluripotent stem (iPS) cells derived by viral transduction of stem cell genes. The cytoplasmic factors present in Xenopus egg extract can alter chromatin structure through a series of DNA and DNA-binding protein changes that lead to expression of early embryonic and developmental genes (Byrne et al., 2003; Kimura et al., 2004; Taranger et al., 2005; Freberg et al., 2007). Therefore Xenopus egg extract system offers an opportunity to identify cytoplasmic factors that have the ability to induce endogenous expression of these genes. Here we report on identification of extract fractions containing sufficient activity to induce expression of embryonic transcription factors, Oct4/Nanog/Sox2, in human dermal fibroblasts and acquisition of developmental potency to cross lineage differentiation boundaries. Our strategies in identification of active “reprogramming activity” components employ an extract fractionation approach utilizing chromatography separation techniques and reconstitution of activity with pools of adjacent fractions and evaluation of the activity by a 2-step bioassay (Step 1 - real-time monitoring of removal of somatic histone H1, and Step 2 - Oct4, Nanog and Sox2 gene activation in transgenic human dermal fibroblasts). Analysis of active extract fractions revealed that both, Xenopus protein and RNAs are required and sufficient for induction of this phenotypic change (Gonda et al., Nat Cell Bio 2003).

1214/B372
Pancreatic Acinar Cells Can Dedifferentiate into Facultative Progenitors.
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Objective: to determine if differentiated cells of the pancreas can dedifferentiate into multipotent progenitor cells. No evidence has been found that the pancreas contains dedicated stem or progenitor cells. However, ligands for the epidermal growth factor receptor (EGFR) induce a dramatic change in the cellular composition of the pancreas, suggesting that progenitor cells may be induced. Methods: Following overexpression of EGFR ligands, most acinar mass is replaced by hyperplastic ductal epithelium. within these hyperplastic ducts are endocrine cells, including insulin-expressing cells. We used genetic lineage tracing to determine the cell of origin for both hyperplastic ducts and their associated insulin cells. Results: We previously demonstrated that acinar cells can rapidly change their identity to ductal cells In Vitro when treated with EGFR ligands. We now show that this transdifferentiation from acinar to ductal identity occurs in vivo. However, acinar cells are limited in their ability to give rise to other cell types. The insulin cells that are found within acinar-derived ductal epithelium do not arise from acinar cells but rather are descendents of preexisting insulin cells. Conclusion: Our work suggests that fully differentiated cells of the pancreas have the ability to dedifferentiate into facultative progenitors, but that these progenitors are restricted to the exocrine or endocrine lineage from which they arose.
1215/B373

Germ Plasm-Like Dot Cells Maintain the Regenerative Function after In Vitro Expansion.
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We have identified a group of E-cadherin, integrin-beta1 and CXCR4-positive small particles from the bone marrow and blood of mice, rats and humans. We termed these particles “Dot cells” due to their morphology in culture. Freshly isolated Dot cells regenerate wounds with less scar and can be cultured without feeder layer cells. We show here that cultured Dot cells have a germ plasm-like morphology but not the eukaryotic cell morphology as we previous thought. They show rapid replication In Vitro and form spheroids when they reach confluence. Dot cells express the germ plasm specific marker VASA, indicating that they are rich in RNA. In addition, they express embryonic stem cell specific markers Oct4, Nanog and Sox-2. When co-present with differentiated cells, Dot cells undergo nuclear programming before becoming eukaryotic cells. Their lineage is determined by the lineage of the co-cultured cells. In Vitro expanded Dot cells that are isolated from albino mice, maintain their wound regenerative activities after intravenous transplantation to wounded black diabetic mice. Dot cells regenerate both epithelial and dermal cells in the wounded sites. The Dot cell-regenerated hair follicles, smooth muscle, and dermal tissues express VASA. Our data demonstrate that Dot cells are the truly regenerative factors. They maintain their regenerative function after In Vitro expansion.

1216/B374

Germline Commitment in Embryonic and Induced Pluripotent Stem Cells.
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Pluripotency effectors OCT4, SOX2 and NANOG suppress differentiation in embryonic stem (ESC) and induced pluripotent (iPSC) cells. Leukemia inhibitory factor (LIF) sustains their pluripotency in culture, in spite of which ESCs still may spontaneously differentiate to the germline. We hypothesize that the germ cell progenitors regulate and preserve their pluripotency through post-transcriptional regulation by, among other proteins, the DEAD-box helicase family. We found that germline proteins DPPA3, IFITM3, DAZL, MIWI and DDX4 are expressed in undifferentiated mouse ESC and iPSC. Ddx4 has a diffuse distribution pattern (LIF+) and aggregates into small clusters around the nucleus during differentiation (LIF-). Ddx4 protein distributes equally to the dividing daughter cells until a germ granule forms. Embryonic bodies of mESCs and hESCs differentiate to follicle-like structures containing presumptive oogenic cells. These cells express Ddx4 protein in nuage/P-granules surrounding a big nucleus. Differentiation toward germline reveals 2+ folds change in germ granule mRNAs (Thap11, Tdrd7, Dazl, Brnuol4, Acr, Hcfc1, Zp3). Mouse iPSCs delay Ddx4 or Dppa3 activation In Vitro compared with ESCs. iPSC-derived EBs retain Ddx4 mRNA expression 3 times less compared with ESC-derived. Although Oct4 expression is down-regulated for iPSCs and ESCs, Sox2 expression remains 70% and higher for studied iPSCs. mESC Ddx4 shRNA knock down (KD) clones retaining 30% of endogenous gene expression did not demonstrate any significant morphological changes on a feeder layer, but partially differentiate on gelatin even under Lif+. We observed reduction in cell growth, proliferation and aggregation ability in KD clones. These clones demonstrate a 2-3 fold increase in Sox2 mRNA expression. We performed mRNA immunoprecipitation (RIP) of DDX4 protein germ granule complex. The ESC RIP sample was 4+ fold enriched in Stau1, Stau2, Oct4, Nanog, Ddx4 and Sox2. Thus, the expression of germline proteins in ESCs or iPSCs might be involved in post-transcriptional regulation of cell pluripotency. We speculate that this evolutionary conserved mechanism ensures early germline specification and differentiation notwithstanding their remaining pluripotency.

1217/B375

A Specific Gene Expression Signature for Determined Asymmetric Self-Renewal by Post-Natal Tissue Stem Cells.
Although asymmetric self-renewal is a defining concept for stem cells in post-natal tissues, it is poorly understood. Generally, asymmetric self-renewal by undifferentiated tissue stem cells is inferred from their production of lineage-specific differentiated cells in stem cell-deficient hosts. Direct analyses of asymmetric self-renewal are infrequent, because of the technical challenge presented by its dynamic and cell-heterogeneous nature. In fact, even the form of asymmetric self-renewal by tissue stem cells In Vivo persists as a matter of uncertainty since the formulation of the tissue stem cell concept nearly half a century ago. Stochastic forms, based on multiple stem cells per tissue unit, are at one extreme, whereas tissue units based on single determined stem cells are at the other. Determined asymmetric self-renewal by individual tissue stem cells is defined by divisions that yield another stem cell and a lineage-committed cell. Two labs (1,2), including this one (2), have demonstrated determined asymmetric self-renewal by individual cells in tissue stem cell-enriched cultures. Previously, we also engineered murine cell lines that undergo single-cell determined asymmetric self-renewal under experimental control (3). Here, we report the use of the engineered cell models to define a specific gene expression signature for cells undergoing determined asymmetric self-renewal. We used Between Group Analysis, a supervised classification method, to evaluate the discrimination power of this “asymmetric self-renewal associated (ASRA)” gene expression signature using public microarray databases for murine tissue stem cell-enriched populations and lineage-committed cell populations. We found that undifferentiated, long-term repopulating, tissue stem cell populations (e.g., long-term hematopoietic stem cells) were consistently associated with determined asymmetric self-renewal, whereas early lineage-committed progenitors, embryonic stem cells, and induced pluripotent stem cells were not. This finding indicates that the ASRA gene signature, and potentially some of the individual genes that compose it, is a promising new resource for direct investigations of asymmetric self-renewal by tissue stem cells.

1218/B376
An Adjustable Protein Expression System and “Pluripotency” Reporters for Reprogramming Factors of Induced Pluripotent Stem Cells (iPSC).
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Reprogramming somatic cells into induced pluripotent stem cells (iPSC) has been accomplished by expressing pluripotency factors. Nevertheless, the kinetics of reprogramming and gene expression requirements are still not fully understood and extensive research is needed with iPSC cells before any potential therapeutic use can be realized. To address this point six reprogramming factors including Oct4, Nanog, Sox2, c-Myc, KLF4, and Lin28 were expressed using the Adjustable Protein Expression System (APEX). The APEX system is based on fusing the open reading frame (ORF) of these six reprogramming factors with a 12 kD (107 amino acid) mutant of the FKBP protein resulting in destabilization of the protein by targeting it for proteasomal degradation. The fusion protein is stabilized by a small, non toxic, membrane permeable FKBP ligand allowing for accumulation of the protein in the cells. It was hypothesized that by adding different amounts of FKBP ligand or a uniform amount FKBP ligand for different periods of time, quantitative control the amount of reprogramming factors could be achieved for optimal induction of pluripotency and subsequent down-stream differentiation of iPSC cells. To test this, five cell-based “pluripotency” factor luciferase reporter constructs were made to measure the biological function of the iPSC related transcription factors. The initial results showed that the fusion proteins from the expression constructs were biologically competent. In addition, the reporter assays quantified varying levels of stabilized protein expression showing that the APEX system enables a rapid and precise control of reprogramming factor levels for iPSC. Significantly, these reporters can be used together as a “pluripotency” marker panel for mechanistic studies as...
well as during screening for additional reprogramming factors and/or small chemical compounds capable of generating iPS cells.

1219/B377  
**Nerve-Like Cells Obtained from Frozen Human Fibroblasts Grown in DMSO Free Nerve Differentiation Media.**  
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DeCoppi et al (2007) demonstrated that amniotic fluid derived stem cells were capable of differentiating into cells of all three germ layers, including nerve cells. We applied these protocols for differentiation of adipose, hepatic, muscle and nerve cells to skin fibroblasts taken from frozen samples varying in ages from the embryo to 96 years of age. These various aged samples had numerous cells with the microscopic appearance of all four cell types after being passed in enriched alpha MEM at least three times and then placed in the appropriate differentiation media. We also prepared these samples for microarray gene expression studies and observed that three of the four tissues-adipose, hepatic and muscle demonstrated distinct gene expression patterns—however, the cells having a nerve morphology did not show any differences in gene expression when compared to undifferentiated cells. The DeCoppi nerve differentiation protocol has DMSO as one of its components. Croft and Przyborski (2006) showed that DMSO can cause the artifactual appearance of nerve like cells in cultures exposed to nerve differentiation media. This prompted us to create a nerve differentiating media that lacked DMSO. These experiments showed that nerve like cells can be seen with this DMSO free differentiation media. They stain positive for Neural Filament-M and Glial Fibrillary Acidic Protein and they also show gene expression profiles different from undifferentiated cells. Some 35 up-regulated and 140 down-regulated genes were seen having at least a two fold different between differentiated and undifferentiated cells—with almost all of the up regulated genes having unknown functions. Further studies to investigate the functionality of these cells are needed.

1220/B378  
**Inhibition of CTP Synthesis Induces Formation of Novel Cytoplasmic Rods and Rings in Cancer as Well as Primary Fibroblasts, Cardiomyocytes, and Stem Cells.**  
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A novel cytoplasmic rods and rings (RR) structure was first identified by human autoantibodies in cancer cells. HEP-2 cells showed 1 to 2 distinct cytoplasmic rods (~3-10 μm in length) and/or rings (2-5 μm dia.). Colocalization studies have shown that RR are not the Golgi complex or any other known cytoplasmic structures. Our previous study has identified a 55kDa protein known as cytidine triphosphate synthetase (CTPS) as a component of RR. This current study was to determine whether functional CTPS is required for the formation of RR and further characterize their presence in other cells including embryonic stem cells (ESC). Indirect immunofluorescence was performed to determine the effect of CTPS inhibitors, 6-diazo-5-oxo-L-norleucine (DON) and acivicin, on the formation of RR using specific human antibodies. Our data showed that both DON and acivicin exhibited dose-dependent induction of RR in ~95% of treated HEP-2 cells compared to untreated cells. Induction of RR was similar with 2mM of either DON or acivicin after 3-4 hours. Knocking down CTPS in HEP-2 cells using siRNA had no affect on RR formation induced by DON or acivicin. The knockdown of CTPS was monitored at >90% using realtime PCR. These results showed that CTPS was not necessary for the formation of RR. Similar observation was observed in other cancer cell lines and including primary cultured fibroblasts, and cardiomyocytes.
Interestingly, RR were detected in untreated, undifferentiated ESC but not in differentiated ESC induced by retinoic acid. Further study revealed that RR-negative differentiated ESCs were capable of forming RR when treated with CTPS inhibitors. In summary, our data has shown the formation of RR is induced by the functional inhibition of CTPS; however CTPS is not a necessary component for RR formation. The expression of RR in cancer cells and ESC suggest their potential roles in cellular proliferation and development.

1221/B379
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Innovative methods for isolation and expansion of pancreatic adult stem cells (PASCs) would be invaluable in the development of cell therapies to treat diabetes. Here we used a transgenic approach for suppression of asymmetric cell kinetics (SACK). We postulated that expression of the XPRT gene (xanthine phosphoribosyl transferase) combined with xanthine-supplementation in the culture medium would allow PASCs to divide symmetrically and expand. After extracting islets and plating them on gelatin-coated dishes in the absence of any cell sorting, we found that only the islets isolated from XPRT-expressing mice and grown in xanthine-supplemented medium gave rise to transferable epithelial cell strains. Clonal strains were established by replating individual colonies that showed varied levels of SACK-responsiveness (SACK-R), defined as the degree to which a cell strain increased its proliferation rate in xanthine-supplemented medium. The clonal strains express cytokeratin-18, PDX-1 and Islet-1 at varied levels, indicating endocrine origin. Subcloning experiments indicate that the strain showing strong SACK-R has a subpopulation of PASCs. These SACK-derived strains form insulin-expressing islet-like clusters on a poly-D-lysine substrate. At early passages (18), after injections in ectopic sites in mice, the strain showing the weakest SACK-R formed tumors, whereas the strain showing the strongest SACK-R remained non-tumorigenic at late passages (45). The former strain also forms pancreatic tumors after intraperitoneal injection, while cells from the latter strain home in the pancreas to form foci after intraperitoneal injection in newborn mice, as demonstrated by fluorescent tagging of the transplanted cells. Long-term (up to 12 months) engraftment is also detected with this last strain. Altogether, our results show that the SACK method allows for the derivation of PASC strains that home in the pancreas and reduces the emergence of transformed variants during long-term propagation. Transposing our SACK method for the derivation of human adult pancreatic stem cells could lead to new avenues in the treatment of diabetes.

1222/B380
Heretofor Unidentified Stem Cells in Human Liver Disease.
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Background: Liver Stem Cells are thought to preside in bile ducts and the canals of Hering. They expand into the liver parenchyma at a time when normal liver cell proliferation is suppressed and liver regeneration is stimulated. Method: In the present study it was found that human cirrhotic livers and hepatocellular carcinoma (HCC) exhibited isolated single hepatocytes scattered stem cells within the liver parenchyma and the HCC. These cells expressed liver stem cell markers. Associated HCC also contained isolated tumor cells which showed the same stem cell markers. The markers used were GST-P and OV-6, identified by immunofluorescent antibody staining, and HGF, EGF, CK19 and H19 identified by RNA-FISH. H19 is a non-coding RNA, which is expressed in most HCCs. Results: Immunohistochemistry and RNA-FISH performed on human livers identified isolated stem cells in liver parenchyma as follows: Precirrhotic ASH or NASH all stained negative for these stem cells. In cirrhotic livers, 12 out of 28 had liver parenchymal stem cells present. In one case of stage III precirrhosis, stem cells were found. In HCCs, 13 out of 15 had stem cells located within the tumor. Double staining for the markers showed colocalisation of the markers in stem cells. Conclusion: Stem cells identified by immunohistochemical markers
(OV-6 and GST-P) and RNA-FISH markers (HGF, EGF, CK19 and H19) were found in the scattered parenchyma of cirrhotic livers and within hepatocellular carcinomas (HCCs) with high frequency, especially in HCCs. Supported by NIH/NIAAA grant 8116.

1223/B381

ABC2 Expression in Placenta-Derived Stem Cells (PDSCs) Represents Sensitive Toxicity for Hepatotoxicants Regardless Hepatic Differentiation.

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Placenta-derived stem cells (PDSCs) are characterized by their capacity to differentiate into hepatic lineages as well as mesenchymal lineages. for this reason, PDSCs have raised great hopes as a source for future cell-based therapy in various degenerative diseases; however, the utility of PDSCs for potential drug candidates has not been extensively studied. The objective of the study is to evaluate their potential as an In Vitro hepatotoxicity model and to elucidate ABCG2 function in stem cells for hepatotoxicity. PDSCs and bone marrow-derived mesenchymal stem cells (BM-MSCs) were cultured with different concentrations of carbon tetrachloride (CCl4, a well-known hepatotoxicant) for 24 hours, 48 hours and 72 hours. After CCl4 exposure, their cell viability and sensitivity for CCl4 treatment were measured by the MTT method. Also, RNA and protein expressions level of ABCG2, which is known for transferring mediator of substrates across the cellular membrane for elucidation of regulatory mechanism of hepatotoxicity were analyzed and the sensitivity to CCI4 in stem cells was analyzed after siRNA of ABCG2 and ABCG2 inhibitor treatment. The cell viability (IC50) in PDSCs was more significantly decreased than it in BM-MSCs for 48 hours and 72 hours (p<0.05). Also, the toxicity of cells exposed to CCI4 before hepatic differentiation was more sensitive than it after hepatic differentiation. Interestingly, the expression of ABCG2 in PDSCs was decreased after hepatic differentiation. Down-regulation of ABCG2 in stem cells by siRNA of ABCG2 and ABCG2 inhibitor treatment induced the sensitivity to CCI4 in PDSCs and BM-MSCs. These results suggest that the sensitivity of PDSC was sensitive than it of BM-MSCs to CCI4 and the sensitivity of cell toxicity was related to ABCG2 expression. Finally, PDSCs expressing ABCG2 may provide useful tools as an In Vitro model of hepatotoxicity as well as toxicity studies.

1224/B382

Sphingosylphosphorylcholine-Induced Expression of Long Pentraxin PTX3 in Human Mesenchymal Stem Cells.

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The PTX3, also called TNF stimulated gene14 (TSG14), is a member of long form of pentraxin proteins and participates in regulation of innate resistance to inflammatory responses. Accumulated evidence suggests a pivotal role of mesenchymal stem cells (MSCs) in tissue remodeling. In this study, we demonstrated that sphingosylphosphorylcholine (SPC) increased expression of PTX3 in human adipose tissue derived stem cells (hATSCs). Moreover, SPC treatment increased secretion of PTX3 protein from hATSCs. In order to elucidate the signaling mechanisms involved in the SPC-induced PTX3 expression, we examined the effects of various inhibitors against SPC-stimulated signaling enzymes. We found that pretreatment of the cells with the Rho kinase inhibitor Y27632 completely abrogated SPC-induced PTX3 expression. However, other pharmacological inhibitors, such as MAP kinase inhibitors (U0126, SB202190, and SP600125) and Ca2+/calmodulin kinase pathway-specific inhibitors (KN93 and W7), had no significant impact on the PTX3 expression. Taken together, these results suggest that SPC induces expression of PTX3 through Rho kinase-dependent mechanism.
Extracellular Matrix and Cell Behavior (1225 – 1248)

1225/B383
Intrinsic Fibronectin Matrix Properties Regulate Embryonic Stem Cell Fate.
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Fibronectin (FN), an extracellular matrix protein that assembles into a fibrillar network, plays a significant role in the development and maintenance of most tissues. As we show here, it’s intrinsic properties can also direct the initial fate choice of embryonic stem cells (ESCs) toward endoderm. As matrix is first secreted, temporal and spatial correlations were found between the expression of FN and GATA-4, an endoderm marker, as well as an inverse correlation with the expression of the self-renewal gene Nanog. During initial commitment, matrix’s fibrillar structure can specifically regulate endoderm development, inducing an 8-fold increase versus conventional ligand coatings. By 2 weeks in culture, ESCs can substantially remodel planar substrates such that their environment is similar to fibrillar matrix and produces similar endoderm upregulation. On the other hand, crosslinked fibrillar matrix, which 10-fold stiffer than uncrosslinked, induced ESCs to significantly upregulate differentiation markers after 2 weeks, suggesting that the temporal sequence of displaying such factors is critical. Detection of these intrinsic matrix properties appears to be dependent on binding of α5β1 integrins to FN. Taken together, our data suggests that matrix assembly and mechanics, when displayed at the right time and place, can induce endoderm expression.

1226/B384
Matrix Stiffness Directs Cytoskeletal Ordering and Nuclear Shape in 2D and 3D.
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It is now generally accepted that the micro-mechanical environment of cells has a significant impact on their behavior. Physical cues such as matrix elasticity can be as important as biochemical cues and can influence complex biological processes including differentiation. For example, human mesenchymal stem cells (hMSCs) from bone marrow have been found to respond to matrix elasticity by differentiation toward various lineages depending on the Young’s elastic modulus (E ~ 1 - 34 kPa) of collagen-coated two dimensional polyacrylamide (PA) substrates. Here, we present a method to finely tune the elasticity of cross-linked hyaluronic acid-based biocompatible hydrogels over a wide, physiologically relevant range and show the applicability for hMSC culture in two and three dimensions, demonstrating that cellular mechanosensitivity is a general feature and is not material dependent. The non-toxic hydrogel system allows for the precise control of the matrix elasticity around embedded cells and also to independently control lower and upper matrix elasticity in a 3D sandwich configuration for the first time. We quantitatively study the organization of the contractile acto-myosin stress fibers of mesenchymal stem cells (hMSCs) in 2D and 3D and find a non-monotonic behavior of the cytoskeletal organization dependent on matrix elasticity. Furthermore, we analyze the morphology of the nucleus and find a non-linear dependence on matrix elasticity that is coupled to the cytoskeletal arrangement illustrating the mechanical link between extracellular matrix and the nucleus. These novel well-defined biocompatible hydrogel environments allow for quantitative dissection of cues from dimensionality and matrix elasticity and open new opportunities for potential in Vivo applications as well as more sophisticated biomimetic extracellular matrix models of mechanically and biochemically heterogeneous environments.

1227/B385
Localized Neurite Outgrowth Sensing through Micro-Patterned Elastic Substrates.
Localized cell responses in areas such as cell-substrate interactions, cell motility, and cell mechanics have provided insights into a diversity of fields over the past decade in areas such as apoptosis, motility, and differentiation. These areas are directly linked to neuroscience as well, where they play critical roles in cell responses including morphogenesis, mechanotransduction, and differentiation. One key component in these interactions are the local elasticity of the substrate as cells are subjected to and also exert mechanical influence, which ultimately affects biochemical signals. To investigate the effects of localized physically related responses in neuroblasts, we developed a novel method to control the substrate microenvironment through generating a polymer composite with alternating elasticities that interact with cells. This technique was developed through fabricating polymeric microchannels using conventional soft lithography and poly(dimethylsiloxane) (PDMS) where localized surfaces had elastic moduli of 800 kPa and 200 kPa adjacent to one another. We then seeded neuroblasts onto the systems and examined their response in terms of outgrowth. After differentiating the neuroblasts, we found that their processes exhibited distinct patterns as they approached and crossed the elastically defined interfaces of the polymer composite. Depending on the location of the soma and the direction of the outgrowth when compared to the elasticity interface, the outgrowths would extend forward in distinct paths including turning back, aligning along, or crossing the elasticity interface boundary. We believe that this approach will enable greater understanding of neural outgrowth, neural regeneration, and mechanobiology as well as provide insight to diseases linked to cell-substrate interactions.

1228/B386

Regulation of Renal Proximal Tubular Cell Differentiation and Morphogenesis by ECM and Substratum Stiffness.

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The signals from extracellular matrix (ECM) are required for cells to execute their physiological properties. Recent studies indicate that substratum stiffness regulates cell behavior, i.e. stem cell fate, cancer cell phenotype, apoptosis and growth factor-induced cell differentiation. Here we explored whether substratum stiffness affected cell differentiation and TGF-β1-induced epithelial-mesenchymal transition (EMT) in proximal tubular cells, i.e. LLC-PK1 cells and primary culture of mice proximal tubular (mPT) cells. TGF-β1-induced EMT could be alleviated by matrigel-contained medium as well as low substratum stiffness in both cells. Interestingly, mPT cells cultured on collagen typeI-coated polyacrylamide gel with stiffness of lower than 2k Pa did not spread out, but maintained cell aggregates structures regardless of the presence of TGF-β1. In addition, low substratum stiffness preserved tubule morphology and differentiation properties to some extent, which was markedly enhanced by matrigel-coated PA gel. Thus, matrigel and low substratum stiffness synergistically regulated differentiation of mPT cells. Moreover, low substratum stiffness not only reduced protein level of Smad3 and ERK, but also downregulated β1 integrin protein and mRNA level. Taken these data together, signals from basement membrane play important role in maintenance of epithelial cells differentiation and prevention of TGF-β1-induced EMT via low substratum stiffness. This study facilitates our understanding of how ECM and substratum stiffness regulates the normal physiological function and the pathological development of renal fibrosis.

1229/B387

Age-Dependent Differences in Mechanical Behavior of Cardiac Fibroblasts.

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Cell-extracellular matrix (ECM) interactions are critical in a number of normal cellular functions including migration, adhesion, differentiation and apoptosis. Cell surface receptor-ECM interactions mediate outside-in and inside-out signaling pathways through which cells perceive and respond to changes in ECM mechanics and deformation. At the cellular level, this adaptation to changing mechanical cues is clearly illustrated in the heart during both development and disease. In the fetal heart the hemodynamic load is relatively low and cardiac fibroblasts produce very little collagen. After birth, the hemodynamic load increases and fibroblasts synthesize and remodel a highly organized collagen-rich ECM. Similarly, fibroblasts in adult hearts respond to changes in tissue mechanics associated with hypertension by depositing more ECM, a cellular response that contributes to cardiac hypertrophy. In this study we directly compared the collagen-remodeling behavior of fibroblasts from normal neonatal and adult rat hearts. The ability of these cells to remodel 2D collagen substrates was examined using digital image correlation strain analyses and 3D collagen gel remodeling was assessed by contraction assays. Local, cell-induced strains on collagen substrates were steady over time and statistically similar between neonatal and adult fibroblasts. In addition, we observed minimal differences in the mRNA expression of collagen receptors (beta1 integrin, DDR1 and DDR2) or alpha-smooth muscle actin between cell types in 2D cultures. In 3D collagen gels, however, neonatal and adult cardiac fibroblasts exhibited marked differences in ECM remodeling behavior. Both cell populations contracted the gels in a time dependent manner, but neonatal fibroblasts produced a significantly greater decrease in gel area compared to adult fibroblasts (p<0.05). These changes in gel contraction were associated with significant differences in the mRNA expression of beta1 integrin, DDR1, DDR2, and alpha-smooth muscle actin. These results suggest that neonatal and adult cardiac fibroblasts have different capacities to remodel their ECM and underscore the importance of using 3D culture systems to examine cell behavior.

**1230/B388**

**The Mechanical Rigidity of the Extracellular Matrix Regulates the Structure, Motility, and Proliferation of Glioma Cells.**

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Glioblastoma multiforme (GBM) is a malignant astrocytoma associated with a median survival time of 15 months, even with aggressive therapy. This rapid progression is partly attributed to the propensity of single tumors cells to diffusely infiltrate the surrounding brain parenchyma, a process that is likely mediated by aberrant interactions between tumor cells and the extracellular matrix (ECM). Here, we show that biophysical cues from the ECM regulate key tumor cell properties relevant to invasion. We cultured a series of human and rat glioma cell lines (U373-MG, U87-MG, U251-MG, SNB19, C6) on fibronectin-coated polymeric ECM substrates of defined mechanical rigidity and investigated the role of ECM rigidity in regulating the structure, migration, and proliferation of tumor cells. on highly compliant ECMs with rigidity comparable to normal brain tissue, tumor cells appear rounded and fail to productively migrate. As ECM rigidity is increased to levels that are expected to match or exceed the secondary structures of Scherer associated with invasion in vivo, tumor cells spread extensively, form prominent stress fibers and mature focal adhesions, and migrate rapidly. Remarkably, cell proliferation is also strongly regulated by ECM rigidity, with cells dividing much more rapidly on rigid than compliant ECMs. Pharmacological inhibition of nonmuscle Myosin II-based contractility blunts this rigidity-sensitivity and rescues cell motility on highly compliant substrates. Collectively, our results provide support for a novel model in which ECM rigidity provides a transformative, microenvironmental cue that acts through actomyosin contractility to regulate the invasive properties of GBM tumor cells. [Ulrich et al., Cancer Res 2009;69(10):4167-74]
Cellular organization within a multicellular organism requires a cell to assess its relative location, taking in multiple cues from its microenvironment. A cell engages ECM and actively probes the matrix, sensing in deformation the elastic resistance that seems to characterize different tissues, and so to assess how far the feedback extends - by analogy to the 'princess and the pea' fairy tale - we have generated substrates of different elasticity and different thickness on top of rigid supports. The elastic properties of our gels are characterized by AFM-based micro-rheology - a tool that probes at the cellular scale, and mesenchymal stem cells (MSCs) are studied because these cells have proven particularly sensitive to matrix elasticity and microenvironment in terms of their adhesion, their morphology, and even - after days - their differentiation. Cell morphology changes generally take hours, and we find that spread area, focal adhesions and cytoskeleton organization of MSCs on thin and soft gels resemble structures in cells on thick and stiff gels. Thickness sensitivity decreases with stiffness, and initial computational modeling of cell and matrix mechanics lends insight into the sub-cellular sensitivity. Furthermore, continuity of deformation from matrix into the cell and around the cytoskeleton-caged and linked nucleus also suggests mechanisms to affect processes such as differentiation. The results ultimately show that even if one's cells are not of royal descent, they seem to feel the difference between stiff or soft and thick or thin surroundings.

Extracellular Matrix generally consists of collagen and fibronectin fibers that interpenetrate non-fibrous and gel-like proteoglycan, with the result being a crosslinked hierarchical matrix possessing complex physical and chemical properties. We sought to address whether cells 'feel' fiber structures distinct from the ligands on natural fibers by creating and culturing cells on a simplified substrate. We embedded synthetic cylinders of controlled nano-diameter, micro-length, rigidity, surface functionalization, etc. in polyacrylamide gels and functionalized the gel surface with homogeneous ligand for cell adhesion. The microelasticity at scales relevant to cell adhesion and contractility of these ECM-mimetic, cylinder-embedded gels (CEGs) was measured by Atomic Force Microscopy (AFM) and found to be only modestly affected by the presence of cylinders. Mesenchymal stem cells (MSCs) were cultured on the gels because these cells and their derived lineages require interactions with ECM for viability and because these cells often encounter fibrous microenvironments in therapeutic applications (eg. infarct scars). Analyses of cell morphology and focal adhesions show that cells on CEGs behave as if these matrices are far stiffer than elasticity measurements would suggest. The effects depend on the elasticity of the gel, which is interesting because in diseases such as osteoarthritis of cartilage, the proteoglycan is often affected more so than the fibers. These results suggest that cells not only sense an average of the substrate stiffness but also can 'feel' and respond to the underlying, fiber microstructure.

Cross-Linked Collagen by Transglutaminase 2: A Promising Biomaterial for Medical Applications.

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Transglutaminases (TGases) are enzymes that catalyze the formation of isopeptide bonds in proteins. So far, eight mammalian TGases has been identified with different functions and tissue specificity. Among them, the tissue transglutaminase (TG2) is the most ubiquitous and plays an important role in the stabilization of the extracellular matrix which is applicable to the stabilization of natural biomaterials, avoiding the toxicity or immunological responses associated with chemical and physical agents. We first demonstrated that cross-linking of collagen affected cell behavior by accelerating fibrillogenesis, altering the morphology and the elastic properties of the collagen fibers and increasing the pore size of the scaffold. These changes also contribute to making the collagen more resistant to cellular protease degradation. Human osteoblasts (HOB) attach, spread and proliferate more on the cross-linked matrix than on native collagen. We also show that the differentiation of HOB is affected by TG2 cross-linking activity, as demonstrated by increase of alkaline phosphatase (ALP) and osteopontin mRNA expression. Use of DNA array and RT real time PCR indicated that β1, β3 integrins, TIMP-1 and -2 were up-regulated in HOB cultured on a cross-linked collagen matrix, while Western blotting showed that the cross-link increased the phosphorylation of Focal Adhesion Kinase (FAK), a key regulator of cell adhesion and spreading. Increased cell adhesion was confirmed by the observation that HOB were more resistant to the RGD-containing peptide inhibition of integrin-mediated cell attachment when cultured on cross-linked collagen. In conclusion, changes in the tensile strength and the likely exposure of hidden cryptic binding sites for integrins in the cross-linked collagen are capable of affecting cell adhesion, spreading, proliferation and differentiation, modifying the expression of molecules involved in these processes e.g. FAK and integrins. Hence, our interest in the cross-link activity of TG2 to alter the matrix proteins and cell behavior at the wound site, especially when considering that TG2 is found in increased amounts in the matrix in many pathological conditions such as dermal scarring and tissue fibrosis.

1234/B392
Mechanical Properties of the Microenvironment Regulate TGF-β-Induced Cell Fate through Akt.
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The transforming growth factor-β (TGF-β) signaling pathway is often misregulated during cancer progression. In early stages of tumorigenesis, TGF-β acts as a tumor suppressor, through control of cell functions such as inhibition of proliferation and induction of apoptosis. However, as the disease progresses, TGF-β switches to promote tumorigenic cell functions, such as induction of an epithelial to mesenchymal transition (EMT) and increased cell motility. Dramatic changes in the cellular microenvironment are also correlated with tumor progression, including an increase in tissue stiffness. Changes in matrix rigidity have been reported to regulate many cell functions, but if and how matrix rigidity controls TGF-β-induced cell fate is unknown. Here we report that matrix rigidity regulates a switch in the cellular response to TGF-β. Mammary epithelial cells cultured on rigid (5 kPa) fibronectin-crosslinked polyacrylamide gels (corresponding to the stiffness of native tumor tissue) exposed to TGF-β were resistant to apoptosis and readily underwent EMT, as indicated by the loss of epithelial markers such as E-cadherin, ZO-1, and cortical actin and the gain of mesenchymal markers such as N-cadherin. In contrast, TGF-β treated cells cultured on a compliant matrix (0.4 kPa, corresponding to normal mammary tissue) rapidly underwent apoptosis, as indicated by the loss of epithelial markers such as E-cadherin, ZO-1, and cortical actin and the gain of mesenchymal markers such as N-cadherin. In contrast, TGF-β treated cells cultured on a compliant matrix (0.4 kPa, corresponding to normal mammary tissue) rapidly underwent apoptosis. Mechanistic studies revealed this switch was controlled by changes in the activity of Akt, another signaling pathway frequently misregulated during tumor progression. In cells cultured on rigid gels, Akt was highly phosphorylated, promoting cell survival and EMT, whereas cell adhesion to compliant gels markedly inhibited Akt activity. Together these findings suggest a novel mechanism whereby the mechanical properties of the adhesive microenvironment regulate TGF-β and Akt signaling to control tumorigenic cell function.

1235/B393
Cell-Matrix Interactions Regulate Platelet-Derived Growth Factor Receptors in Mesenchymal Stem Cell Migration.
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Human adult mesenchymal stem cells (MSCs) derived from bone marrow have the capacity to self-renew and to differentiate into a variety of cells and tissues. They may leave their niche to migrate to remote tissues and play a critical role in wound repair and tissue regeneration. A major goal in utilising stem cells is to define how MSC fate is controlled by the pericellular extracellular matrix (ECM) and soluble factors that partially constitute tissue-specific niches. Integrin receptors mediate cell adhesion to surrounding ECM, and can also modulate growth factor receptor signalling and cellular responses. The objective of this study was to investigate molecular relationships between integrins and platelet-derived growth factor receptor (PDGFR) tyrosine kinases, which are crucial for the selective expansion and recruitment of undifferentiated mesenchymal cells. MSC adhesion to different ECM ligands with distinct integrin ligation profiles, differentially influenced PDGFR-β phosphorylation. In the absence of growth factor ligand, adhesion to fibronectin (but not laminin) induced tyrosine phosphorylation of PDGFR-β, and this response was mediated by integrin α5β1 but not αvβ3. Cell type specific responses were also noted for differentiated mesenchymal cells such as smooth muscle cells. Co-immunoprecipitation analysis showed association between integrin subunit α5 and PDGFR-β in MSCs adherent to fibronectin. Inhibition studies revealed that integrin α5 activity and PDGF-BB additively stimulated PDGFR-β phosphorylation, leading to MSC migration towards fibronectin. Immunofluorescence microscopy showed that tyrosine (Y1021) phosphorylated PDGFR-β co-localised with integrin α5 in migratory cells, in a tidemark of focal adhesion structures. These findings provide novel insights how the ECM regulates cross-talk between growth factor tyrosine kinase receptors and integrins, and thereby regulates MSC fate. This research was funded by the Medical Research Council (UK).

1236/B394 ABSTRACT WITHDRAWN

1237/B395 Cellular Fibronectin Promotes Hepatic Stellate Cell Migration via a Switch from A5β1 to A9β1 Signaling.
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The goal of our study was to determine how cellular fibronectin (cFN) influences the behavior of hepatic stellate cells (HSC), the major fibrogenic cell type in liver fibrosis, and to define whether this requires signaling through integrin α5β1 or integrin α9β1. Methods: Primary HSC were isolated and cultured on polyacrylamide supports coated with plasma fibronectin (pFN) or cFN. Cell morphology and focal adhesions were examined by confocal microscopy. Chemotaxis was assessed using transwell migration assays, and adhesion was studied using a spinning disc assay. Results: HSC cultured on pFN-coated polyacrylamide supports underwent myofibroblastic differentiation, demonstrating a triangular shape and prominent α-smooth muscle actin (SMA)-containing stress fibers. In contrast, HSC cultured on cFN-coated supports demonstrated decreased incorporation of α-SMA into stress fibers, more lamellipodia, and smaller but more numerous focal contacts, suggestive of a more motile phenotype. This motility difference was confirmed by transwell migration assays, in which HSC cultured on cFN, as opposed to pFN, had greater chemotaxis toward serum. As determined by immunoblotting and real-time PCR, HSC expressed the classic fibronectin RGD-binding integrins, α5β1 and αvβ3, and the alternative integrin α9β1, but not α4β1. By confocal microscopy, α9β1 was localized to the lamellipodia of migrating cells, whereas α5β1 was restricted to mature focal adhesions and colocalized with vinculin. Furthermore, blocking or knocking down α5β1 completely inhibited adhesion to pFN but only partially inhibited adhesion to cFN, indicating binding via non-α5β1-integrins. Conclusions: We have demonstrated that HSC grown on cFN have markedly different morphology, migratory ability, integrin expression, and integrin localization than those grown on pFN. These results
suggest that cFN enhances HSC migration by inducing a switch from α5β1 to α9β1 as the dominant fibronectin-binding integrin. Our data suggest that cFN and integrin α9β1 may have previously unappreciated roles in cell migration and may facilitate HSC chemotaxis after liver injury.

1238/B396
Laser Inactivation Protein Patterning of Cell Culture Substrates.
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Micropatterned substrates are emerging as powerful tools for basic studies of cell function for more technologically oriented applications. Here we describe a highly flexible and facile new approach for patterning biomolecules based on laser inactivation of proteins adsorbed to a cell culture substrate. We show that a focused laser can be used to achieve dose dependent inactivation of proteins adsorbed to a substrate, and that by translating the laser across the substrate arbitrary protein patterns can be produced. Substrates made this way can be used to influence the response of a wide range of cell types, and are suitable for controlling cell shape as well studying the influence of protein organization on cell function. Because the inactivation is dose dependent, gradients of almost any form can be produced. Further, because the focal position can be changed during translation, laser inactivation patterning can be used produce patterns on topographically complex surfaces as well as on the inside of translucent objects (such as small capillary tubes). Substrates can be patterned while the protein is maintained fully hydrated, to prevent denaturation of sensitive molecules. These capabilities add substantially to the arsenal of tools available to the cell biologist to produce engineered microenvironments.

1239/B397
Layer-By-Layer as a Reservoir for Controlled Delivery of BMP-2 to Cells.
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Efficient delivery of growth or survival factors to cells is one of the most important long term challenges of current cell-based tissue engineering strategies. Extracellular matrix acts as a reservoir for a number of growth factors via interactions with its components. In the field of nanobiomaterials, layer-by-layer thin films are emerging as a new and versatile technique to functionalize the surfaces of biomaterials as well as to prepare films having well defined functionalities (in terms of structure, topography and bioactivity) that can be used to understand cellular behaviors and to orient cell fate. Here, we show that poly(L-lysine)/Hyaluronan layer-by-layer films (1) can serve as a reservoir for localized BMP-2 delivery at the cell/matrix interface. The advantage of presenting a growth factor associated with the matrix is to drastically potentiate its effect. Without BMP-2 in the films, cells cultured on to films of increased stiffness exhibit a stiffness-dependent differentiation in myotubes. In the presence of BMP-2 in the films, myoblasts are induced to differentiate into osteoblasts in a dose dependent manner. We proved that the bioactivity of BMP-2 loaded films is due to a direct contact of cells with the films. Importantly, the BMP-2 containing films could sustain three successive culture sequences while remaining bioactive, confirming the positive effect of BMP-2 immobilization as compared to BMP-2 added in solution. These films may find applications in the local delivery of immobilized growth factors for tissue engineered constructs (4)and they may also be used as tools to understand growth factor interactions with cell receptors. (1) Schneider, et al.Polyelectrolyte multilayers with a tunable Young’s modulus : influence of film stiffness on cell adhesion. Langmuir, 22: 1193-1200, 2006. (2) Ren, K. et al. Polyelectrolyte multilayers of controlled stiffness modulate myoblast differentiation. Adv. Funct. Mat.18, 1-12, 2008. (3) Boudou, T. et al; Multiple functionalities of polyelectrolyte multilayer films : new biomedical applications. Advanced Materials; review, in press july 2009.

1240/B398
Lung Vascular SMC Differentiation Controlled by Prx1-Dependent ECM Property.
Homeobox genes encode highly conserved transcription factors that control tissue patterning and morphogenesis yet little is known about their roles in lung vascular development. Recently, however, we demonstrated that the paired-related homeobox gene, Prx1, is essential for lung vascular development and its deficit is due to failed extracellular matrix (ECM) expression. In support of this, in this study, we found that Prx1-null lungs possessed unorganized elastic ECM accompanied with less deposition of tropoelastin, fibrillin-1 & -2 and associating growth factor TGF-beta in and around vessels of Prx1-null lungs. In addition, vascular SMC differentiation was suppressed in Prx1-null lungs. Based on these, we hypothesize that Prx1 plays a critical role in promoting mesenchymal precursor cell differentiation to a vascular SMC phenotype depending upon both the biochemical (TGF-beta signaling) and biophysical (ECM compliance) properties of the elastic ECM. To test the hypothesis, we used a stiffness controlled 2D culture system with combination of TGF-beta stimulation, as well as the newly developed 3D culture system utilizing the decellularized fetal lung scaffolds from Prx1-wild type and -null mice. on the 2D culture system, precursor cells cultured on Prx1-null ECM stiffness substrates showed low level of SMC differentiation even with TGF-beta stimulation whereas the cells cultured on the wild type stiffness intensely differentiate in SMC. Furthermore, in the 3D decellularized scaffolds system, precursor cells cultured within Prx1-wild type lung scaffolds colonized and organized appropriately, whereas Prx1-null scaffolds failed to achieve this state. This deficit is also accompanied by an inability to differentiate into SMC. Blocking of TGF-beta function inhibited SMC differentiation in cells cultured on wild type scaffolds. Collectively, these results indicate that Prx1 indirectly regulates lung vascular development via its ability to promote the maturation of biophysical and biochemical ECM properties that favors lung precursor cell differentiation.

1241/B399
Synergy in the Effects of Tenascin-C and TGF-β1 on Human Mesenchymal Cells.
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Wound contraction through myofibroblast generation is a critical element of tissue repair after injury, and myofibroblast persistence has been associated with pathological fibrosis and scar contractures. TGF-β1 is a key regulator of myofibroblast formation and fibrosis, which includes assembly of a fibronectin matrix. How signals from this growth factor are modulated by signals from the extracellular matrix (ECM), however, are not well understood. Tenascin-C is a large extracellular glycoprotein that is up-regulated during wound healing but whose role in this process remains poorly characterized. To explore how these two proteins contribute to cell behavior during wound healing, we investigated the impact of exogenous tenascin-C and TGF-β1 on human dermal fibroblasts and adipose-derived mesenchymal stem cells using an In Vitro model of myofibroblast phenotype induction. Expression of the myofibroblast-associated protein marker α-smooth muscle actin (α-SMA) was examined by western blot and immunofluorescence in the presence of either TGF-β1 or tenascin-C or both proteins combined. Effect on cell function was evaluated using matrix contraction assays, which measured the ability of cells to contract a fibrin-fibronectin matrix. Compared to the presence of either TGF-β1 or tenascin-C alone, cells cultured in the presence of both proteins exhibited a dramatic increase in α-SMA expression by 48 hours. The combined presence of TGF-β1 and tenascin-C also had a greater impact on the ability of cells to contract a fibrin-fibronectin matrix compared to either one alone. These results suggest that tenascin-C enhances the impact of TGF-β1 on cell behavior and support the concept that ECM proteins act synergistically with growth factors to regulate wound cell responses during tissue repair.
Inflammation is a critical response to injury and infection. However, inappropriate inflammation is the major hallmark of autoimmune diseases, including rheumatoid arthritis (RA), and is a key contributing factor to the metastatic tumor environment (Mantovani, Science: 2009). The immune response is triggered by invading pathogens: pattern recognition receptors (PRRs) recognise distinct microbial products and in response stimulate the expression of pro-inflammatory genes. Endogenous molecules generated upon tissue damage in response to injury or infection act as ‘danger signals’ that also stimulate inflammation (Matzinger, Science: 2002). Provocation of inflammation by these molecules, whilst ensuring an effective response to tissue injury in the absence of infection, also contributes to maintaining chronic inflammation by invoking a perpetual destructive cycle. As such these molecules are increasingly being targeted as therapeutic candidates in inflammatory diseases (Midwood et al., Current Drug Targets: 2009). We have identified tenascin-C, an ECM glycoprotein that is specifically induced at sites of inflammation including in RA and in the tumor stroma, as endogenous activator of the immune response. Mice with targeted deletions in tenascin-C are protected from prolonged inflammation and joint destruction In Vivo (Midwood et al., Nature Med.: 2009). Tenascin-C mediates it pro-inflammatory effects by activating a specific PRR; toll-like receptor (TLR) 4. TLR4 is also activated by bacterial cell wall lipopolysaccharide (LPS). The mechanism of LPS activation of TLR4 requires two specific co-receptors, CD14 and MD-2. However, it is not clear how endogenous molecules activate TLR4. We have shown that tenascin-C induces the activation of significantly different pro-inflammatory signalling pathways downstream of TLR4 compared to LPS, and that tenascin-C does not require the co-receptors CD14 or MD-2. By further understanding how endogenous ligands stimulate the immune response we hope to find ways to limit excessive inflammation in autoimmune diseases and in tumors without compromising host response to infection.

1243/B401
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Cells respond to the stiffness of their microenvironment by altering their morphology and, in some cases, even their gene expression profile [Engler et al, Cell 126, 677]. We study the behaviour of human mensechymal stem cells (hMSC) cultured on thin highly ordered collagen films, and we show that hMSC are sensitive to nano-mechanical properties of collagen coated substrates. Cell morphology, cytoskeleton organization, and differentiation are evaluated in response to different collagen crosslinking agents. Cells pull on the collagen films, and their ability to deform the collagen fibrils is greatly influenced by the films’ mechanical properties. Mechanically anisotropic native collagen films promote strong polarization and orientation along the highly aligned fibrils. Transglutaminase cross-linked films lose their distinct anisotropic mechanical properties and give a very different cell response. In comparison to crosslinked polyacrylamide gels coated with collagen-ligand, the morphology of the cells on the native and pure collagen films resembles that of cells on soft gels (myogenic phenotype) while cells on cross-linked collagen films appear more like cells cultured on stiff gels which can promote osteogenic differentiation. Cells cultured for two weeks on transglutaminase crosslinked collagen fibrils indeed express the osteogenic marker CBFα1 in contrast to cells cultured on noncrosslinked collagen films. Atomic Force Microscopy techniques are used to evaluate local topography as well as the mechanical properties of the cells and their surroundings at high spatial resolution. The AFM stylus is used also to deform the fibrils, mimicking cellular processes of collagen remodelling. Crosslinked collagen films require forces which are at least twice as high for similar plastic deformations of native collagen films. We
also measure the elasticity or effective tension of live cells in response to the different collagen films and conclude that cells stiffen considerably after two weeks on the stiff crosslinked collagen films. The results show that cells are highly sensitive to the crosslink-based nanomechanics of their matrix.

1244/B402
Robust Characterization of Hydrogel Microstructure Using Laser Tweezers Particle Tracking and Confocal Reflection Imaging: Implications for 3D Cell Cultures.
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Hydrogels are commonly used as extracellular matrix mimetics for applications in tissue engineering and increasingly as cell culture platforms in which to study the influence of biophysical and biochemical cues on cell function in 3-D. Using standard methodologies, the bulk mechanical properties of a variety of hydrogels have been characterized and correlated to cell function. However, very few studies have focused on linking the microstructural mechanical properties of hydrogels with cell function in 3-D, in part because of a lack of appropriate techniques. Here we have utilized a laser tracking system based on passive optical micro rheology instrumentation to characterize the microstructure of viscoelastic fibrin clots. Trajectories and mean square displacements were observed as PEGylated 1 µm microspheres diffused within confined pores created by the protein phase of fibrin hydrogels. We address the importance of surface modification to minimize non-specific interaction of microspheres with proteins for micro rheology and similar methods, which rely on measurements of their displacement. Complimentary confocal reflection imaging revealed microstructures comprised of a highly heterogeneous fibrin network with a wide range of pore sizes. As the protein concentration of fibrin gels was increased, our quantitative laser tracking measurements showed a corresponding decrease in both the size and range of pores with greater resolution and sensitivity than conventional imaging techniques.

1245/B403
Heparan Sulfate-Dependent Interaction of Syndecan-1 with EMMPRIN on the Cell Surface Suppresses Tumor Cell Migration: Identification of the Binding Site of Emmprin to Syndecan-1 Heparan Sulfate.
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Objective: EMMPRIN (extracellular matrix metalloproteinase inducer) is a membrane-bound glycoprotein with two extracellular loop domains. We have previously revealed that EMMPRIN enhances tumor cell migration through an active site in the second loop domain (termed EM9). On the other hand, EMMPRIN has been reported to bind to various functional molecules through extracellular loop domains, which is closely associated with the physiological and pathological functions of EMMPRIN. However, the molecular mechanisms of EMMPRIN-mediated tumor cell migration are still unclear. In the present study, we examined the association of Syndecan-1, a membrane-bound heparan sulfate proteoglycan, with EMMPRIN-mediated tumor cell migration in human tumor cells. Methods: The expression of EMMPRIN and Syndecan-1, and their interaction on the cell surface of human uterine cervical carcinoma SKG-II, fibrosarcoma HT-1080, glioma U251, and melanoma Mewo cells were investigated by immunocytochemical, Western blot, and co-immunoprecipitation analyses. Cell migration was measured by scratch-wound assay. Results: EMMPRIN and Syndecan-1 were co-localized on the cell surface of all tumor cells tested. Syndecan-1 formed a complex with EMMPRIN through its heparan sulfate, whereas the enzymic deletion of N-glycosylation in EMMPRIN did not alter the interaction between Syndecan-1 and EMMPRIN. A synthetic EM9 peptide interfered with the EMMPRIN-Syndecan-1 interaction and thereby increased SKG-II cell migration. In addition, cell migration was dose-dependently augmented by administering an antibody against EM9 of EMMPRIN. Furthermore, not only
enzymic deletion of heparan sulfate in Syndecan-1 but also the transfection of Syndecan-1 siRNA facilitated SKG-II cell migration. Conclusions: These results provide novel evidence that Syndecan-1 negatively regulates EMMPRIN-mediated tumor cell migration by a heterogeneous complex formation in that heparan sulfate of Syndecan-1 interacts with the EM9 region in the second loop domain of EMMPRIN. Finally, these findings may provide new insights into the development of antitumor-metastatic strategies that target the Syndecan-1/EMMPRIN complex.

1246/B404
Neutrophil Elastase Is Expressed in Pulmonary Artery Smooth Muscle Cells and Contributes to Pulmonary Vascular Disease in MHV-68-Infected S100A4 Overexpressing Mice.
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Obstructive neoimal pulmonary arterial (PA) lesions in patients with PA hypertension have been related to heightened expression of S100A4, a metastasis gene, and to a prior infection with human herpesvirus (HHV-γ). Our laboratory reported that mice overexpressing S100A4, but not control C57 wild-type (WT) mice, develop similar PA neoimal lesions following infection with murine HVγ (M1-MHV-68). The development of PA neoimal lesions is preceded by pulmonary serine elastase activity and associated with on-going elastolysis in PAs characterized by fragmentation of elastic laminae and proliferation of PA smooth muscle cells (SMCs). Previously, we implicated vascular elastase activity in the development of PA lesions, because the enzyme generates pro-mitogenic and pro-migratory elastic peptides and releases growth factors. We hypothesized that heightened expression of an endogenous vascular elastase was responsible for PA neoimal formation in the murine model described above. Our goals in this study were (i) to determine the extent to which serine elastase activity was required for neoimal formation in S100A4 mice infected with MHV-68 and (ii) to identify the nature of the enzyme. We show that administration of an inhibitor of serine elastase, elafin, at 2mg/kg/day for the first month following MHV-68 infection of S100A4 mice infection, reduced serine elastase activity and PA neoimal lesion development. To isolate and characterize the serine elastase activity, we utilized a FLAG-immunoaffinity column conjugated to 6kDa FLAG-tagged elafin. The elastase activity was isolated from lung homogenates of MHV-68-infected S100A4 mice. Mass spectrometry of elafin-bound proteins identified two peptides with complete homology to neutrophil elastase reflecting the only serine elastase in the homogenate. We then showed that neutrophil elastase mRNA was expressed in central PAs from S100A4 mice and from cultured human PA SMCs but not human PA endothelial cells. Furthermore, immunohistochemistry revealed that PA smooth muscle cells express neutrophil elastase. We therefore implicate PA SMC production of neutrophil elastase in the pathogenesis of obstructive pulmonary arterial neoimal lesions.

1247/B405
DDR1 and DDR2 Affect Platelet-Collagen Interactions.
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Collagen type 1 is the major component of the extracellular matrix (ECM) in the tunica adventitia of the vessel wall. Damage to the vascular wall leads to exposure of fibrillar collagen type 1 to the circulating platelets. It has been well established that platelets adhere to and become activated by this fibrillar collagen. The formation of collagen fibrils is modulated by several collagen binding proteins, including Discoidin Domain Receptors 1 and 2 (DDR1/DDR2) as established previously by our group [Agarwal, et al. JMB 367(2007) 443-55; Mihai, et al. JMB 385 (2009) 432-45; Blissett et al., JMB 385 (2009) 902-11]. However, very little is known on how platelet-collagen interaction is affected by collagen binding proteins. This work investigates how changes in collagen fibrillogenesis induced by DDRs influences platelet activation. Platelet rich plasma (PRP) was removed from healthy adult volunteers. It was confirmed that platelets do not contain DDR1
and/or DDR2. Collagen type 1 (0.5 to 10 ug/ml) was incubated with and without the purified extracellular domains of DDR1, DDR2, or a control protein, TrkB (in a 5:1 ratio by weight) and was used as an agonist for platelet aggregation. DDR1, DDR2, or TrkB alone failed to affect platelet aggregation. The time to platelet aggregation in the presence of DDR1 or DDR2 with collagen was markedly increased as compared to pure collagen at identical concentration for the same donors. These results suggest that DDRs interfere with the formation of fibrillar collagen, which in turn influences the activation of platelets. The novel insights provided by our results may have potential implications in wound healing, thrombosis, plaque formation, and specific leukemia’s where collagen binding proteins such as the DDRs are up-regulated.

**1248/B406**

**A Novel Gene Associated with Cellular Adhesion in Dictyostelium Discoideum.**

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We have identified a novel gene in Dictyostelium discoideum that presents a cell-surface adhesion defect when disrupted. The gene was first identified during a Restriction Enzyme Mediated Insertion (REMI) screen. The disruption in D. discoideum gave rise to cells lacking the ability to remain attached, under normal handling conditions, to the surface of a petri dish. The disruption showed no effect on cell growth, either in static or in suspension cultures. The REMI insertion was found to be 245bp upstream of a predicted gene, DDB_G0270794, described in the genomic database (dictyBase). The gene was cloned into an over-expression vector and cell lines generated. In initial experiments cells were grown as attached cultures for 4-5 days and then exposed to trypsin for a set time with agitation, followed by two separate washes in PBS, with agitation. Each wash was carefully removed and the cells were counted and cell number adjusted for volume. Cell numbers were then converted to percent of total cells for each experiment. Following the initial trypsin wash the percent adherent cells for the parental (DH1) cell line was 53.3± 8.23% while the over-expression (JC) cell lines were 73.2± 6.95% adherent. After the last wash the cells remaining on the dish had a mean of 14.4± 4.8% adherent DH1 vs. A mean of 61.5± 8.92% adherent for JC cells. In a modification of the experiment, cells were allowed to attach for only 3-hours vs. 4-5 days. Surprisingly, in all cases the cells were more adherent after the 3-hour attachment period compared to the 4-5 day attachment period. However, consistent with above results, the JC cells were significantly more adherent as compared to the DH1 cells (68.5± 9.68% for the DH1 cells vs. 98.8± 0.774% for the JC cells). Therefore, DDB_G0270794 represents a novel gene that contributes to the overall cellular adhesion in D. discoideum.

**Extracellular Matrix and Cell Signaling (1249 – 1273)**

**1249/B407**

**Rac1 Is a Substrate for Non-Receptor Tyrosine Kinases FAK and Src.**

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Integrins regulate cell behavior through recruitment and activation of signaling proteins at sites of cell-matrix adhesion. Two of these signaling elements, Src and FAK, are non-receptor cytoplasmic tyrosine kinases that may act cooperatively during the regulation of cell spreading and migration. We have previously shown that FAK can tyrosine phosphorylate PIX with subsequent induction of Rac1 activation, increased Rac1 targeting to focal adhesions, and improved cell spreading. The present study demonstrates that FAK and Src directly phosphorylate Rac1 at Y64. A Y-F (tyrosine to phenylalanine) point mutation at this position significantly diminished tyrosine phosphorylation of Rac1 by FAK and Src in vitro, indicating that Rac1 Y64 is the major phosphorylation target for these tyrosine kinases. Furthermore, both Y-F
and Y-D (tyrosine to aspartic acid) point mutations at position 64 altered cell spreading. MEF and HUVEC expressing Rac1-Y64F mutant spread better than the wild type controls, whereas cells expressing Rac1-Y64D mutant show only limited spreading. As compared with wild type Rac1, Rac1-Y64F also displayed increased GTP-binding, increased association with PIX, and reduced binding with RhoGDI. Taken together, these data define a novel mechanism for the regulation of Rac-1 activity by non-receptor tyrosine kinases, with consequences for membrane extension.

1250/B408
FAK Activity Is Essential for Developmental and Cytokine-Stimulated VCAM-1 Expression.
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Loss of focal adhesion kinase (FAK) yields embryonic lethal phenotypes and cell proliferation defects. As FAK functions both as a cytoskeletal scaffold and integrin-associated signaling protein, knockouts do not provide mechanistic insights in distinguishing these features of FAK action. To determine the role of FAK activity during development, a kinase-inactive knockin mutation (R454) was introduced into mice by homologous recombination. FAKR454/R454 mice were embryonic (E) lethal at E9.5 with yolk sac primary capillary plexus defects and failure in allantois-chorion fusion. Primary FAKR454/R454 mouse embryonic fibroblasts (MEFs) proliferated in vitro, but exhibited enhanced focal adhesion formation and motility-polarity defects associated with elevated RhoA GTPase activity and loss of p190RhoGAP tyrosine phosphorylation upon cell binding to fibronectin. Gene array analyses revealed 5-fold reduction in vascular cell adhesion molecule 1 (VCAM-1) mRNA expression in FAKR454/R454 embryos which may account for the lack of chorion-allantois fusion during development. Autonomous VCAM-1 expression in FAKWT/WT but not FAKR454/R454 MEFs suggested that FAK activity is indispensible for promoting VCAM-1 gene expression. The inflammatory cytokine tumor necrosis factor α (TNFα) stimulates VCAM-1 expression and pharmacological inhibition of FAK activity blocks TNFα-stimulated VCAM-1 expression in human umbilical vein endothelial cells (HUVECs). Whereas TNFα-stimulated NF-κB activation is normal under FAK kinase-inhibited conditions in MEFs and HUVECs, TNFα-stimulated extracellular-regulated kinase (ERK2) activation is dependent on FAK activity. These studies provide genetic support for the importance of FAK activity in promoting directional cell motility and uncover a unique FAK to ERK2 signaling linkage needed for developmental- and inflammatory cytokine-associated VCAM-1 expression.

1251/B409
Targeting of TIAM1 to Focal Adhesion through Talin for Rac1 Activation and Cell Migration.
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Cell migration is necessary for developmental morphogenesis and tumor metastasis. A typical migrating cell has front-rear polarity with a single leading edge at the front and a tail at the rear. The Rho family GTPases play critical roles in directional migration. Rac1 activation generates a vectorial protrusion that is stabilized by adhering extracellular matrix through integrins. Integrin-ligations, in turn, activate Rac1. This positive circuit between Rac1 and integrin is critical for cell polarity at the front of migrating cells. However, it remains largely unknown how adhesion signals activate Rac1 at the specialized region. We herein identified Tiam1, a Rac1 specific GEF, as a novel binding protein to Talin. Head domain of Talin associates with the middle part of Tiam1 which includes PDZ domain and its flanking region. In migrating cells, Tiam1 targets to focal adhesion preferentially at the front in a manner dependent on Talin. Rac1 activation upon integrin-ligation to fibronectin requires Talin and Tiam1. Furthermore, the Talin-binding to integrin and Tiam1 facilitates cell spreading as well as migration. Thus, the targeting of Tiam1 to adhesion sites appears to account for asymmetric Rac1 activation at the front of polarized migrating cells.
1252/B410
The Effects of Rock Isoform Knockdown on Fibroblast Contractility.
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The Rho kinases (ROCKs) mediate contraction in non-muscle cells such as fibroblasts by regulating the activity of myosin phosphatase and therefore the phosphorylation state of Myosin II regulatory light chain. Fibroblasts contain two homologous isoforms of rho kinase: ROCK 1 and ROCK 2, but the individual roles of each isoform are not well understood. The aim of this study was to investigate the specific roles of the ROCK isoforms in fibroblast contraction and proliferation. We silenced each ROCK isoform in human foreskin fibroblasts (HFFs) using shRNA, and used transduced fibroblasts to produce three-dimensional engineered tissues by embedding the cells in a type I collagen matrix. Contractile force of HFF engineered tissues was quantified in response to stimulation with 1µM lysophosphatidic acid (LPA) using the Palpator™. To measure the effects of ROCK isoform knockdown on cell growth, we performed assays using 3-(4,5-Dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT). We were able to reduce ROCK 1 protein expression in HFFs by 73 percent, and ROCK 2 expression by 49 percent, as confirmed by Western blot. LPA induced force development in engineered tissues with ROCK 1 suppression was comparable to controls, whereas force development in engineered tissues with ROCK 2 suppression was reduced by 50 percent. These results suggest that ROCK 2 is the predominant isoform associated with contraction of non-muscle tissues. Interestingly, ROCK 1 expression was also reduced by shRNA constructs targeting ROCK 2, indicating that ROCK 2 may regulate expression of ROCK 1. Cell growth was decreased following transduction with shRNA lentivirus, but did not appear to depend on ROCK 1 protein expression. The growth rate of cells transduced with shRNA targeting ROCK 1 and control cells transduced with shRNA targeting eGFP was similarly reduced. These results indicate that ROCK 1 may not have an important function related to cellular proliferation.

1253/B411
Cell Shape Regulates SRF-Mediated Transcription to Control Proliferation.
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Changes in cell shape can regulate numerous cell functions including differentiation and proliferation. While restricting cell spreading decreases proliferation, it is unclear how cell shape mediates the nuclear transcriptional changes underlying proliferative control. Analyzing microarray data obtained from cells spread to different degrees on micropatterned substrates revealed several transcription factors with significant expression changes or with significant binding site overabundance in the gene promoters, one of which was serum response factor (SRF). SRF controls the expression of several genes necessary for proliferation, including the immediate early genes, and therefore could be a key transcriptional link between cell shape and proliferation. Real time PCR and luciferase assays demonstrated that unspread cells decreased expression of SRF-target genes, but only when the promoter contained the full serum response element (SRE), which includes the CArG box (binds SRF) and the Ets site (binds ternary complex factors (TCFs)). Genes lacking the Ets site were not regulated by cell shape, suggesting that the Ets site confers shape-dependent regulation of SRF. Because map kinases (MAPKs) are primary regulators of TCF activity, we examined their role in mediating shape-regulated SRF signaling. Unexpectedly, ERK was not required for SRF activity. However, inhibition of p38 MAPK rescued both SRF activity and proliferation in unspread cells, while inhibition of JNK decreased SRF activity and proliferation. Furthermore, cells in which TCF (Elk, Net, or Sap) expression had been stably knocked down showed proliferation defects. ChIP analysis showed that changes in cell shape shifted the binding of the three TCFs to the promoter of the immediate early gene egr1 differently. To identify whether such shifts apply to certain subsets of TCF target genes, we are currently using ChIP-sequencing methods to characterize which TCF-regulated proliferative genes are controlled by cell shape changes. These data highlight the pivotal role that cell shape...
plays in the transcriptional regulation that controls growth and proliferation and points to a novel mechanism by which this occurs.

1254/B412
Extracellular Regulation of Growth Factor Signaling by Fibrillin Microfibrils.
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Genetic evidence in humans and mice as well as biochemical data implicate the microfibrillar network in the regulation of TGF-β and BMPs in the extracellular space. In order to test whether interactions with fibrillins are required In Vivo for appropriate regulation of BMP signaling, we examined tissues in Fibrillin-2 (FBN2) -/- mice. These mice are born with forelimb contractures which resolve over time. We found that the differentiation of the skeletal muscle is delayed in the FBN2 -/- mice. This was accompanied by reduced muscle mass, altered muscle architecture, and perturbed BMP signaling shown by increased phospho smad 1/5/8 staining at P0. at P8, when the contractures resolve, the muscle mass has normalized and the muscle architecture looks much improved. In order to test whether abnormally activated BMP signaling is responsible for the observed skeletal muscle phenotype, we treated the diseased FBN2 -/- forelimbs with Noggin protein in organ culture. This significantly improved the muscle architecture, as shown by H&E stain. Moreover, we were able to induce a similar muscle phenotype by treating control limbs with BMP7 in organ culture. BMP expression at P0 was not altered, suggesting that the delay in skeletal muscle differentiation is due to abnormal activation of BMP signaling, rather than to increased BMP expression. Therefore, we tested the effects of knocking down FBN2 during In Vitro differentiation of C2C12 mouse myoblasts into myotubes. Results showed increased BMP activity in the cell culture media, further corroborating that FBN2 is controlling BMP activity. Biochemical analyses of FBN2 -/- forelimb muscle tissue showed delayed expression of myosin heavy chain 8 (MYH8), a marker for perinatal muscle differentiation linked with Trismus-Pseudocamptodactyly syndrome, which is characterized by distal arthrogryposis. This connection could be a crucial link in explaining the underlying biochemical mechanisms causing limb contractures in the absence of a fibrillin-2 containing matrix. All together, we show for the first time that Fibrillin-2 is required to limit BMP activity. This might be exerted through proper targeting of BMPs and also through sequestration of BMP activity by fibrillin.

1255/B413
In Vivo Analyses of Microfibril Assembly and Growth Factor Signaling.
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Deficiency of either fibrillin-1 or fibrillin-2 results in dysregulated TGFβ (1) or BMP (2) signaling. In order to test hypothesized molecular mechanisms by which fibrillin-1 controls growth factor signaling, we have generated two new Fbn1 mutant mouse models. To test whether sequestration of the large latent TGFβ complex requires interaction with fibrillin-1, the LTBP binding site in fibrillin-1 (3) was deleted by homologous recombination. In contrast to all other homozygous Fbn1 mutant mice, homozygous Fbn1 mutant mice with an in-frame deletion of exon 7 (H1Δ, hybrid 1 deletion) live well beyond the early postnatal period and assemble microfibrils that appear to be normal. Homozygous H1Δ mice are, however, small, suggesting a deficit of TGFβ signaling. To test whether activation of TGFβ and BMP signaling is associated with fragmentation of microfibrils, Fbn1 was truncated and an eGFP tag was brought into frame behind exon 32, using homologous recombination and Cre-lox technology. Heterozygous GT-8 (green truncated from founder 8) mice live and breed but develop aortic aneurysm and dissection. Homozygous GT-8 mice die in the early postnatal period (P9-P24). Both heterozygous and homozygous mice assemble eGFP-truncated fibrillin-1 into microfibrils. However, incorporation of
the mutant fibrillin-1 targets the microfibrils for degradation. BMP signaling is activated in these mice when microfibril fragmentation becomes apparent. In contrast, activation of TGFβ signaling appears later and is accompanied by accumulation of large amounts of collagen. Based on these studies, we conclude that fibrillin-1 is required for appropriate targeting and sequestration of the large latent TGFβ complex and that proteolysis of microfibrils, initiated by assembly of mutant fibrillin-1 into microfibrils, is accompanied by activation of growth factor signaling. Thus, a major physiological function of the fibrillin microfibril scaffold is to target and sequester growth factors.


1256/B414
The Rho GEF, GEF-H1, Is Regulated by 3-Dimensional Matrix Density.
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Cell interactions with the extracellular matrix (ECM) have a profound influence on carcinoma progression, invasion, and metastasis. We and others have established that breast epithelial cells sense and respond to the stiffness of their extracellular matrix environment using the small GTPase Rho, however the exact signaling mechanism has not been identified. In addition to Rho, focal adhesion kinase (FAK) has been linked to mechanosensing, and studies from our lab have revealed FAK as a key mediator of breast tumor progression. We have found that mammary specific deletion of FAK results in tumors that are neither locally invasive nor metastatic. Expression analysis from FAK-/- tumors demonstrated that a number of Rho regulatory molecules were regulated by FAK. Of particular interest, mRNA for the Rho GEF, GEF-H1 (Arhgef2) was significantly decreased in FAK-/- mammary tumors. While relatively little is known about GEF-H1, it has been implicated in the crosstalk between microtubules and the actin cytoskeleton, positioning it as a potential regulator of Rho-mediated contractility. While it is established that Rho activity is regulated by matrix stiffness, Rho regulatory molecules that modulate signaling in 3D matrices, and specifically in response to changes in matrix stiffness, have yet to be identified. Using a nucleotide free version of RhoA, we have now demonstrated that GEF-H1 activity is regulated by matrix density. Additional studies are underway to establish the role of GEF-H1 in Rho activation, breast cell invasion and carcinoma progression.

1257/B415
Filamin Association with β1 Integrins Regulates Invasion into 3D Collagen Matrices.
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Physical and mechanical properties of the extracellular matrix (ECM) can regulate different aspects of epithelial cell behavior such as migration, proliferation, and morphogenesis, and can have a significant impact on cancer progression. The mechanisms whereby the matrix elicits these behaviors, however, remain unclear. We have recently shown that the density of 3D collagen gels, when properly balanced with cell contractility through Filamin A-β1 interactions, is an important regulator of ductal morphogenesis. In addition, we have previously demonstrated that collagen realignment at the tumor/stromal interface occurs concurrently with cell invasion out of tumors, suggesting that collagen alignment facilitates invasion. Using a cell-seeded plug assay in 3D collagen gels to further investigate the effects of collagen alignment on cell migration, we found that cells preferentially migrate within regions of aligned collagen, and that migration is increased in cells that have more β1-filamin interactions. at higher collagen gel density, this increase in migration compared to wild type cells is more pronounced, and may reflect a greater ability for cells with increased β1-filamin interactions to generate aligned collagen fibers, particularly in high density gels. Additionally, we find that enhanced filamin binding to β1 alters
the strategy of migration from primarily individual chains of cells to large sheets or clusters of cells. These findings suggest that the cells' level of contractility not only affects migration speed, but also dictates migration phenotype. Ongoing experiments are currently aimed at determining the effects of β1-filamin interactions on the extent of collagen alignment, in order to further understand the underlying mechanisms by which cells migrate in 3D matrices.

1258/B416

The Missing Link: HEF1-Aurora A-HDAC6-Cortactin Pathway in Invadopodia Formation and Metastatic Progression.

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Recent laboratory studies have linked HEF1 overexpression to melanoma and breast cancer metastasis in animal models, with elevated HEF1 expression corresponding with increased cellular invasion in vitro. However, the molecular mechanism and signaling pathways utilized by HEF1 to drive tumor invasion and metastasis are unknown. We have shown previously, that HEF1 binds and activates the mitogenic kinase Aurora a (AurA) to stimulate cell proliferation, and AurA activity is significantly increased in advanced metastatic cancers. One target of AurA is the unconventional histone deacetylase HDAC6. HDAC6 activity is elevated in motile and invasive cells, and AurA phosphorylation of HDAC6 activates HDAC6 deacetylase activity towards several cytoskeletal proteins. One protein deacetylated by HDAC6 is the actin-regulatory protein cortactin (CTTN), an event that increases cortactin binding to filamentous (F-) actin and promotes tumor cell migration. Cortactin is a critical component of invadopodia, membranous carcinoma cell protrusions that control extracellular matrix degradation of the basement membrane by concentrating and secreting matrix metalloproteinases (MMPs). The ability of cortactin to interact with F-actin is an absolute requirement for invadopodia formation and function. In our work, we have shown that HEF1 regulates CTTN’s ability to bind F-actin through several mechanisms: HEF1 directly binds to CTTN to scaffold invadopodia complex and promotes AurA-dependent activation of HDAC6 to deacetylate CTTN. Depletion or inhibition of AurA kinase or HDAC6 by specific shRNAs or small molecule inhibitors, respectively limits invadopodia formation and invasion of metastatic breast cancer cells. Therefore, a HEF1-AurA-HDAC6-CTTN pathway possesses all of the attributes of a key candidate signaling route utilized by breast cancer cells to acquire an invasive and metastatic phenotype. The results of our study could provide rationale for the use of recently developed AurA and HDAC6 therapeutic inhibitors in the treatment of patients identified with early HEF1 overexpression.

1259/B417

Bit-1 Activates an NFkB Cell Survival Pathway in an Integrin/PI3K Dependent Manner.

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Cell survival signals are modulated by many different receptors including receptor tyrosine kinases and integrins. Integrins themselves can influence cell survival through more than one pathway. Apoptosis that results from a loss of integrin-mediated cell attachment to the extracellular matrix is called anoikis. We have previously shown that Bit-1 is an effector of anoikis upon placing cells in suspension in serum containing media. The anoikis function of Bit-1 is only counteracted by integrin-mediated cell attachment. The objective here was to explore integrin regulation of Bit-1 in attached cells. We show that in attached cells knockdown of endogenous Bit-1 by siRNA decreased cell survival in serum free conditions and re-expression of Bit-1 abrogated this effect. ShRNA reduction of Bit-1 promoted serum-deprivation mediated apoptosis in the cells attached to fibronectin but not in cells attached to collagen IV. Attached cells expressing endogenous Bit-1 were more resistant to staurosporine induced apoptosis compared to siRNA knockdown counterparts. In attached cells Bit-1 protected from serum deprivation-
mediated apoptosis and staurosporine induced mitochondrial apoptosis by up-regulating NFkB activity and subsequently bcl-2 gene transcription. Bcl-2 protein expression and protection from apoptosis under serum-free conditions correlated with bcl-2 transcription. This Bit-1-mediated regulation of bcl-2 is FAK and PI3K/AKT-dependent. When cells lacking Bit-1 were plated on fibronectin FAK phosphorylation was significantly reduced as detected by Western blot. Bit-1 is predominantly cytoplasmic and pro-apoptotic when no integrin is bound and is targeted to the mitochondria and anti-apoptotic when specific integrins are ligated. Our findings suggest an integrin-controlled pathway in which Bit-1 is, at least in part, responsible for the survival effects of cell-ECM interactions.

1260/B418
The Role of Aurora A and HDAC6 in Breast Cancer Invasion.
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Cancer progression ultimately correlates with an increase in invasion and metastasis. In order to invade, cancer cells often form protease-enriched ventral membrane protrusions called invadopodia. The protein cortactin is a core component of invadopodia and is required for matrix degradation. The activity of cortactin is tightly regulated by HDAC6 (Histone Deacetylase 6), a unique tubulin deacetylase. HDAC6 has recently been shown to regulate cancer cell motility through the deacetylation of cortactin, which is necessary for cortactin to bind to F-actin and form invadopodia. Over-expression of HDAC6 was found in various types of tumors and was shown to be necessary for cell transformation. Broad spectra HDAC inhibitors are currently in clinical trials where they show great potential by inhibiting tumor growth and in some cases inducing tumor regression; but the molecular mechanisms of these inhibitors are largely unknown. We previously identified mitotic Aurora a (AurA) kinase as a potent HDAC6 activator and demonstrated its involvement in the deacetylation of tubulin. In the current study we tested the hypothesis that the inhibition of HDAC6 or AurA could potentially inhibit invadopodia formation and invasion due to the inability of acetylated cortactin to bind F-actin. Using several invasive breast cancer cell lines and clinically relevant AurA and HDAC6 inhibitors we demonstrate that inhibition of AurA prevents HDAC6 phosphorylation and decreases its activity. We measured invasion by counting functional invadopodia, as determined by the observation of gelatin matrix degradation. We found that when either AurA or HDAC6 is inhibited, breast cancer cells form fewer invadopodia and are less invasive. Based on these findings, we conclude that the use of AurA and HDAC6 inhibitors would be potentially beneficial for treatment of late stage breast cancers, where there is increased invasion and metastatic potential.

1261/B419
Heparan Sulfate Proteoglycan Mediates Secretion of April from Neutrophils.
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A proliferation-inducing ligand (APRIL) is a member of the tumor necrosis factor (TNF) superfamily that is expressed largely by tumor and immune cells. However, in breast tumor stromal tissues, neutrophils have been implicated as the major source of APRIL. Certainly, we found that APRIL is expressed in neutrophils that were cocultured with either T47D or Hs578T breast tumor cells which do not express APRIL. Interestingly, we observed a time-dependent increase in APRIL secretion by neutrophils that were cocultured with the breast tumor cells, suggesting a stimulatory effect by the tumor cells. However, culturing breast tumor cells on a transwell insert placed above a neutrophil layer did not elicit APRIL secretion from neutrophils. Similarly, conditioned media from breast tumor cells did not cause neutrophil secretion of APRIL, indicating that cell-cell communication or a contact-dependent secretory mechanism is involved. Since heparan sulfate proteoglycans (HSPGs) are abundant cell-surface molecules that are
associated with the extracellular matrix, and have been shown to mediate cell-cell communication and signaling, we sought to determine whether the HSPG analogue, heparin, could induce APRIL secretion from neutrophils. Indeed, neutrophils showed APRIL secretion dose-response to heparin. Conversely, heparinase treatment of breast tumor cells prior to coculture with neutrophils significantly reduced the latter’s ability to secrete APRIL, implying an HSPG-mediated mechanism of APRIL secretion from neutrophils. Given that HSPG is a presumed APRIL receptor, particularly in non-immune cells, we propose a dual role of HSPG for APRIL: as receptor and signaling molecule for APRIL secretion. Thus, HSPG may modulate the level and effect of neutrophil-derived APRIL in the breast tumor microenvironment.

1262/B420
Arsenic Induces Cyclooxygenase-2, Vascular Endothelial Growth Factor and MMPs in Association with Tumor-Associated Invasion and Angiogenesis in Human Uroepithelial Cells.

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Arsenic is widely distributed in the nature. Its contamination to the water source is considered as one of the health hazards for human being. In the southern west coast of Taiwan, an endemic area of arsenicism, is found high incidence of urothelial carcinoma. According to the literature, exposure of arsenic will lead to angiogenesis, and angiogenesis is essential for tumor growth, invasion, and metastasis. on the other hand, a proinflammatory factor, cyclooxygenase-2 (COX-2), which usually is not expressed in normal tissue, but overexpressed in malignant tumor, and it is related to oncogenesis, tumor invasion and metastasis. In endemic area of arsenicism, it has been shown that exposure of arsenic induce COX-2 and vascular endothelial growth factor (VEGF) overexpression. However, the study of these factors in the mechanism of arsenic tumorigenesis and angiogenesis is limited. So, we intend to investigate the mechanism of angiogenesis in the arsenic oncogenesis, and try to find out the relationship between dose of arsenic treatment and the COX-2 expression, as COX-2 is an important factor in oncogenesis of uroepithelia. In this study, we use immunocytochemistry and Western blotting to investigate the expression of COX-2, VEGF, MMPs, and plasminogen activators(uPA) of uroepithelial cell line under different doses of arsenic exposure. Our results revealed that in low dose ( 4 μM) of arsenic treatment, overexpression of COX-2 and VEGF protein expression and cellular transformation of urothelia is noticed. on the other hand, in high dose (10 μM) exposure, the arsenic will cause cytotoxicity to the urothelia and decreased of COX-2 and VEGF protein expression, suggested that the arsenic treated cell underwent apoptosis. In addition, the MMP-2 and -9 protein expression, and cell invasion ability were also increased in low dose of arsenic exposure, but decreased in high dose. The uPA protein expression was increased in dose-dependent manner. In conclusion, our study demonstrated the relationship between arsenic exposure and its molecular effects to the uroepithelia.

1263/B421
Crucial Role of Vinexin for Keratinocyte Migration In Vitro And Epidermal Wound Healing In Vivo.

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In the process of tissue injury and repair, epithelial cells rapidly migrate and form epithelial sheets. Vinexin is a cytoplasmic molecule of the integrin-containing cell adhesion complex localized at focal contacts in vitro. Here, we investigated the roles of vinexin in keratinocyte migration in vitro and wound healing in vivo. Vinexin knockdown using siRNA delayed migration of both HaCaT human keratinocytes and A431 epidermoid carcinoma cells in scratch assay, but did not affect cell proliferation. Induction of cell migration by scratching the confluent monolayer culture of these cells activated both EGFR and ERK, and their inhibitors AG1478 and U0126 substantially suppressed scratch-induced keratinocyte migration. Vinexin knockdown in these cells inhibited the scratch-induced activation of EGFR, but not that of ERK, suggesting that vinexin promotes cell migration via activation of EGFR. We further generated vinexin (-/-) mice and isolated their keratinocytes. They similarly showed slow migration in scratch assay. Furthermore, vinexin (-/-) mice exhibited a delay in cutaneous wound healing in both the back skin and tail without affecting the proliferation of keratinocytes. Together, these results strongly suggest a crucial role of vinexin in keratinocyte migration in vitro and cutaneous wound healing in vivo.

1264/B422
**PI3-K/Akt/JNK/NF-κB Pathway Regulates MMP-9 Expression in Intact Amniotic Membrane-Expanded Human Limbal Epithelial Cells.**

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Purpose: Limbal epithelial cells expanded on amniotic membrane (AM) are capable of reconstructing the ocular surface with limbal stem cell deficiency (LSCD). Previously, we have demonstrated the role of matrix metalloproteinase-9 (MMP-9) in intact AM (amniotic epithelial cells preserved)-expanded limbal epithelial cells. In this study, we sought to elucidate the signaling pathways mediating MMP-9 expression in this model. Methods: Corneoscleral buttons from human donor eyes were cut into 1.5 x 2 x 3 mm³ pieces and cultured on intact AM for 3 weeks. Different pharmacological inhibitors for mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3-K) and nuclear factor-κB (NF-κB) pathways were added from day 14 for another week to detect their effects on outgrowth as well as MMP-9 expression in this model. The extent of outgrowth was monitored by microscope and calculated by computer software. Expression and regulation of MMP-9 during such expansion was studied by gelatin and casein zymography, in situ casein zymography, reverse transcription-polymerase chain reaction (RT-PCR), and immunofluorescent staining. Results: MMP-9 was preferentially expressed at the leading edge of limbal outgrowth as evidenced by in situ zymography. Both PI3-K and MAPK cascades are involved in limbal epithelial outgrowth on AM. However, only the c-Jun N-terminal kinases (JNK), and PI3-K/Akt pathways participated in MMP-9 regulation at both transcriptional and translational levels. Besides, NF-κB nuclear translocation at the migration front was noted during such expansion, and JNK and PI3-K inhibitors prevented p65 phosphorylation and nuclear translocation. Helenalin, a specific NF-κB inhibitor, not only retarded the limbal outgrowth but also attenuated MMP-9 expression in limbal epithelial cells. Finally, the fact that JNK activity was significantly inhibited by PI3-K inhibitor disclosed that PI3-K/Akt signaling was upstream of JNK pathway in regulating MMP-9 expression in this model. Conclusion: Both MAPK and PI3-K signalings are required for limbal outgrowth on intact AM, however, only the PI3-K/Akt/JNK-mediated pathway regulated MMP-9 expression through activation of transcriptional factor NF-κB in this model.

1265/B423
**Molecular Alterations of Human Prostate Stromal Gene Expression Programs Caused by Cellular Senescence: Potential Roles in Prostate Neoplasia and Therapy Response.**

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As a stress and possibly an age-related process, cellular senescence safeguards against oncogenesis by suppressing, essentially irreversibly, cell proliferation. Though senescence has beneficial anticancer effects, increasing lines of evidence indicates that senescent cells can have deleterious effects that might contribute to age-related pathologies. We used a diverse array of senescence-inducing factors on PSC27, a primary normal human prostate stromal cell line, and compared their abilities to alter the expression of senescence- and proliferation-related genes. Most senescence inducers caused DNA damage and elevated transcripts encoding extracellular proteins, many of which comprise a senescence-associated secretory phenotype (SASP). The upregulated proteins included matrix metalloproteinase (MMP) activity, as determined by zymography. The gene expression changes induced by genotoxic stress in cultured cells are consistent with those induced by DNA damaging chemotherapy in epithelial tumor cells In Vivo after clinical treatment. To understand how these gene expression changes are regulated, we investigated the downstream effects of the cyclin-dependent kinase inhibitor (CDKI)-p16, a critical tumor suppressor protein that maintains the senescence growth arrest, and the function of NF-kB, a major regulator of gene expression programs associated with mammalian aging and genotoxic stress. We also evaluated the potential relevance of a stromally secreted factor, Wnt16. Our data confirmed that p16 is a pivotal senescence effector, and showed that enhanced p16 expression in stromal cells can promote epithelial growth in culture. The data also demonstrated that NF-kB is not the sole mediator of the SASP: preventing its nuclear localization can only partially prevent the SASP phenotype. Finally, overexpression of Wnt16 in stromal cells robustly stimulated cell proliferation, migration and invasiveness, activation of the Wnt/β-catenin signaling pathway and chemoresistance of prostate epithelial cells.

1266/B424
Lacritin's Heparanase-Dependent 'Off/On Switch' Cell Targeting Mechanism in Epithelia Requires Serines 23 and 24 but Not Serine 14 in Human Syndecan-1.
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Lacritin is a 12.3 kD prosecretory mitogen whose C-terminus targets an unidentified site within syndecan-1 (SDC1)'s N-terminal 50 amino acids. Ligation selectively initiates epithelial NFATC1 and mTOR-dependent mitogenesis (Wang et al, JCB '06). Targeting is via a heparanase-dependent off/on switch (Ma et al, JCB '06), suggesting either that SDC1’s heparan sulfate (HS) chains sterically block core protein binding or that the stubs following heparanase deglycanation contribute to a hybrid stub/core protein binding motif. To address this question, a series of human SDC1 and lacritin N-terminal truncation and single or double point mutants were developed, expressed in suspension culture of 293-6E cells and assayed in pulldown experiments. Suspension culture significantly improved the extent of SDC1 glycanation, presumably on serines positioned at amino acids 14, 23 and 24 from the N-terminus of the mature protein. Truncation of 50, but not 20, amino acids from mature SDC1’s N-terminus abrogated binding to lacritin. This was confirmed by point mutational analysis in which SDC1 point mutant S14A bound lacritin, but not S23/24A. Since active heparanase is required for lacritin binding, the implication is that binding is contributed by heparanase-generated HS stubs at serines 23 and 24 (but not 14), in keeping with the inability of lacritin to pulldown SDC1 ectodomain generated in bacteria. Several small SDC1 peptides were employed as soluble inhibitors. Peptides with sequence length spanning SDC1’s N-terminal amino acids 20-30 and 20-40, but not 1-20 and 30-50 significantly attenuated lacritin binding. Lacritin is thought to interact with SDC1 via the hydrophobic face of a C-terminal amphipathic alpha helix. Indeed, binding was unaffected by truncation of up to 75 amino acids from lacritin N-terminus, and partial alteration of the hydrophobic face (S108/109A) significantly lowered affinity for SDC1. These data tentatively suggest lacritin’s C-terminal amphipathic alpha helix targets a hybrid SDC1 binding site that appears to consist of heparanase-generated stubs and associated core protein as part of a novel approach to the renewal of some germative and non-germative epithelia. Supported by NIH EY013143 (to GWL).
1267/B425
Acute Response of Cardiac Fibroblasts to a Disturbed Stress Field: Genomic Analysis of Physiological and Pathological Stress Regulators.
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Organs and tissues function under various levels of tension as part of their normal physiology and development. Most tissues actively produce contractile force to deform, as well as are deformed and stretched passively. In general, the degree of stretch ranges from 5 to 15% in any one direction. Tissues and organs in a normal physiological state establish a homeostasis of three-dimensional (3D) tension field, depending on the direction and degree of a stretch within their microenvironments. Pathological damage to the tissues and organs can alter the 3D tension field, thus contributing to alterations in physiological growth and cellular function. To mimic acute disturbance of the 3D tension field in tissues in vivo, we generated an In Vitro system by which tension fields are applied to an engineered tissue in two-dimensions at various time intervals to mimic acute disturbances in the contractile environment of the heart. The 3D orientation of cells sensing tension in natural tissues was also reconstituted with freshly isolated neonatal rat cardiac fibroblasts (6k cells/ml) and a collagen matrix (1mg/ml). In the newly damaged heart, fibrotic areas or even interstitial fibroblasts might sense the disturbances in applied forces to directly affect the remodeling process. To investigate the molecular signals that might emanate from these stretch-sensitive fibroblasts in the heart, we compared gene-expression profiles of rat fibrotic tissues among non-stretched controls, continuous (48 hrs) uni-directional stretched (15%), and 48 hr uni-directional stretched followed by an acute (4 hrs) stretch in the perpendicular direction of prior stretching. The 48 hrs of stretch aligned the fibroblasts in a direction parallel to the stretch and narrowed the tissue-width ~50% more than that of non-stretched controls. The acute stretch did not change the tissue shape significantly further. We will analyze the details of differentially expressed genes among the different sample groups.

1268/B426
Redox Regulation of α5β1 Integrin Stimulated MMP Production in Chondrocytes.
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Excessive matrix metalloproteinase (MMP) production is a key mechanism by which cartilage extracellular matrix destruction occurs during the development of arthritis. Signaling pathways initiated through the α5β1 integrin, which result in increased production of MMPs, have previously been shown to require the presence of reactive oxygen species (ROS). Stimulation of the α5β1 integrin with fibronectin fragments (FN-f), a physiological stimulus found in arthritic cartilage, results in a significant increase in intracellular ROS levels in chondrocytes. The objective of the current study was to determine the source of ROS and the mechanism by which FN-f triggers the ROS production necessary for stimulating MMP expression. Intracellular ROS levels were measured through the detection of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCFDA). Primary human chondrocytes were pretreated with chemical inhibitors of NADPH oxidase, mitochondrial respiratory chain complex I, 5-lipoxygenase, Rac and Rho and then stimulated with FN-f. The DCFDA assays showed that only inhibition of 5-lipoxygenase or Rac was able to decrease the FN-f stimulated intracellular levels of ROS. Examining the effects of inhibition on the α5β1 integrin signaling pathway, we found that the phosphorylation of MAP kinases and c-jun was significantly decreased when 5-lipoxygenase and mitochondrial complex I were inhibited. Rac inhibition did not have an effect on MAP kinase signaling. Furthermore, FN-f stimulated MMP expression was decreased upon inhibition of 5-lipoxygenase, Rac, and mitochondrial complex I. Together, these results suggest that 5-lipoxygenase may be important for early ROS production upon FN-f stimulation of the α5β1 integrin signaling pathway and downstream MMP production. In contrast, Rac seems to be mediating ROS production for an alternate MMP stimulating pathway that is also initiated by FN-f.
Further investigation of the source and mechanism of ROS production will help to clarify the redox regulation of MMP production in the development of arthritis.

1269/B427
**Regulation of EGF-Dependent Fibronectin Fibrillogenesis by Galectin-3 and Tyrosine Phosphorylated Caveolin-1.**
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In murine mammary epithelial cancer cells, fibronectin fibrillogenesis is dependent on beta1,6-acetylglucosaminyltransferase V (Mgat5) activity and interaction of its β1,6GlcNAc-branched N-glycan products with galectin-3 (Lagana et al., Mol Cell Biol. 26(8):3181-93, 2006). Galectin-3 lattice formation at the cell surface promotes EGF signaling (Lajoie et al., J Cell Biol. 179(2):341-56, 2008) and acts with phosphorylated caveolin-1 to increase focal adhesion turnover and cell migration (Goetz et al., J Cell Biol. 180(6):1261-75, 2008). We show here that long term treatment of EGF stimulates fibronectin fibrillogenesis. Disruption of the galectin lattice by galectin-3 siRNA, by blocking N-glycan processing with swainsonine or by competitive inhibition of galectin binding, prevents EGF-dependent fibrillogenesis. EGF stimulates Cav1 phosphorylation and using siRNA targeting Cav1 as well as Cav1 mutants preventing (Y14FCav1) or mimicking (Y14DCav1) its phosphorylation, we demonstrate that phosphorylated Cav1 is required for EGF-dependent fibrillogenesis. Expression of Y14DCav1 mutant induces fibronectin fibrillogenesis independently of EGF stimulation, but dependent on integrity of the galectin lattice. Finally, specific inhibition of the Rho/Rho kinase pathway by Y27632, but not of the PI3 Kinase and ERK pathways by LY294002 or U0126, prevents EGF-dependent fibrillogenesis. We propose that EGF receptor activation induces fibronectin fibril assembly through Cav1 phosphorylation and Rho pathway regulation. Galectin lattice is required downstream of phosphorylated Cav1 identifying a novel role for phosphorylated Cav1 in the crosstalk between EGF receptor signaling and cell-matrix remodeling which might participate in tumor cell migration. Supported by CIHR grant MOP-43938.

1270/B428
**Chondrogenic Differentiation of Umbilical Cord Derived Stem Cells Under Hypoxic Conditions.**
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Osteoarthritis is a degenerative joint disease characterized by the breakdown of a joint’s cartilage. Loss of articular cartilage is the primary cause of joint dysfunction and subsequent disability. Stem cell based therapies have become attractive models for treatment because once articular chondrocytes have lost their functional ability, they cannot be replaced in adults. Therefore, tissue engineering focused on cell-based therapeutics may provide an avenue to repair damaged extracellular matrix (ECM), and contribute a chondroprotective effect, preserving the remaining native cartilage. We have previously showed umbilical cord derived stem cells (UCSCs) possess mesenchymal stem cell (MSC) characteristics, including: A stable phenotype in culture, high expansion capabilities, and a multi-lineage differentiation potential. The goal of the current study is to assess the chondrogenic capacity of UCSCs under physiological oxygen concentrations. Thereby analyzing the true potential of characterized mesenchymal-like stem cells within the chondrogenic lineage. Full-term umbilical cord samples were digested using Collagenase enzyme, and cells were harvest within 6 hours. Once harvested, the standard pellet assay was followed and UC cell pellets were contained under either 2% (low oxygen) or 20% (environmental oxygen) conditions for different lengths of time. Immunocytochemistry was also used to characterize the effect of ambient oxygen concentration on UC cells based on their expression of surface proteins. After subjecting UC cells in pelleted form to hypoxic conditions,
they appear to become more chondrocyte-like, in that they show enriched extracellular matrix, a larger overall volume and more vibrant histology patterns. The UC-pellet microenvironment induces chondrogenic differentiation of UCSCs as evidenced by Safranin O staining. In addition, immunocytochemistry revealed an alteration in some endothelial-linked protein markers at low oxygen, but no change in MSC marker content. In sum, this demonstrates the ability for UCSCs to respond to stimuli within the chondrogenic lineage; illustrating the importance of environmental conditions on the In Vitro culturing environment

1271/B429
Implication of Rmnd5 E3 Ubiquitin Ligase Activity as a Mediator of Morphological Activities of Muskelin and RanBP9.
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Muskelin and RanBP9 are conserved, co-associated intracellular proteins whose knockdown phenotypes in mammalian cells demonstrate functional roles in cell morphology regulation. RanBP9 also interacts as a scaffold with the cytoplasmic domains of multiple adhesion and tyrosine kinase receptors and mediates association with a widely-expressed complex of proteins characterized by shared LisH and CTLH motifs. A homologous complex in budding yeast functions in proteosomal and vacuolar degradation of specific target proteins through a RING domain E3 ubiquitin-protein ligase, GID2p. Selective protein degradation is an important regulatory mechanism in cells, and aberrations contribute to human disease. We are investigating the hypothesis that mammalian muskelin and RanBP9 also interact with LisH/CTLH complex components and that the complex has E3 ligase activity. By co-immunoprecipitation methods, we identified pairwise physical association of muskelin and RanBP9 with the LisH/CTLH proteins TWA1, Maea and Rmnd5, also association of TWA1 with Maea. Domain requirements for association of muskelin with TWA1 are identical to those for RanBP9 binding, suggesting that RanBP9 links muskelin to LisH/CTLH complex. All proteins within the complex are nucleocytoplasmic proteins, with Maea most highly concentrated in the nucleus and muskelin and RanBP9 being mostly cytoplasmic. By immunofluorescence, cellular localisations of multiple components of the complex are altered under conditions of proteosome inhibition and many become concentrated in aggresome-like puncta; in the case of Rmnd5, localisation was not changed by inhibition of calpains or lysosomal enzymes. Ubiquitinylation of Rmnd5 and TWA1 was detected in proliferating cells. Like GID2p, Rmnd5 contains an RING domain and has E3 ligase activity. These findings implicate Rmnd5 and the regulation of stability of Rmnd5 target proteins as a mechanism for mediating activities of muskelin and RanBP9 in matrix-dependent cell spreading and actin cytoskeletal organisation.

1272/B430
Soluble Egg Shell Membrane Improved Human Skin: From Asian Wisdom to Cell Biology.
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Eggshell membrane (ESM) is the double thin membrane in egg shell of Gallus gallus domesticus, which was written in “Bencao Gangmu” (1578), and have been even now used among Sumo wrestlers to have good healing effect on the recovery from burns, cuts and various diseased conditions. Recently, it has been reported that ESM is also effective for decubitus in Japan. Moreover its hydrolyzed soluble eggshell membrane (SEM) has been used as cosmetics and
supplement material. The aim of this study is to determine the effect of the SEM on the hydration and elasticity in women's skin and its mechanism of the skin improvement. (Exp.1) Thirty female volunteers aged between 20 and 65 were divided into two groups of 15. Subjects of each group applied the cream and moisturizer twice a day for 12 weeks on forearm and upper arm: one a preparation with 1% SEM, and the other without SEM and the capacitance and mechanical property in the application areas of the subjects were measured. The skin hydration in SEM group and control group, and net-elasticity in SEM group significantly improved after the application period. The increase of skin hydration in SEM group was more than control group. (Exp.2) to investigate basic cellular function of SEM, we used a novel biomaterial and a kind of 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer, PMBN, which contains monomer with active ester group as a component. Not only PMBN does inhibit non-specific interaction with biomolecules, but it is a bio-conjugate MPC polymer which can immobilize specific proteins on the tissue culture dish. As a result of binding SEM to tissue culture dish via PMBN, the gene expression of hyaluronan synthase2 increased with dose-dependent manner of SEM in human dermis fibroblasts. These results suggest that SEM application to women’s skin improves the cuticular condition by dermal activation.

1273/B431
Differentiation of Corneal Epithelial Cells on Nanopatterned Surfaces.
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A fundamental question in cell biology is how extracellular surface topography regulates cell behavior. Our group has fabricated polyurethane substrates patterned with topographic features of differing types and dimensions, and has shown that biomimetic topographic features modulate corneal epithelial cell orientation, adhesion, migration and proliferation. We have utilized an immortalized human corneal epithelial cell line (hTCEpi) that exhibits an alignment response on nanopatterned surfaces similar to primary human corneal epithelial cells (HCEC), when cultured in EpiLife medium. Using this model cell line, we have looked at changes in cellular differentiation on nanogrooved surfaces ranging from 400 nm to 4000 nm pitch (pitch = groove width + ridge width). Expression levels of differentiation markers, including aldehyde dehydrogenase (ALDH3), keratin 12 and keratin 14 were assayed using quantitative PCR. These data suggest that the level of hTCEpi cellular differentiation increases with increasing pitch size. Previous data from our group has suggested that the nanoscale topographic features of the substratum constrain focal contact architecture, resulting in altered signaling and cellular responses. These studies suggest that changes in the expression of key cellular components may alter the response of cells to nanopatterned surfaces, indicating possible signaling pathways that are important for corneal epithelial cell growth and differentiation.

Integrins I (1274 – 1287)

1274/B432
Reconstitution of Integrin Activation In Vitro.
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Marked increases in integrin binding affinity (‘activation’) are important in the polarization and pathfinding of migrating cells. Talin binding to the integrin β cytoplasmic domain is a final common step in activation; however, recent data has suggested that additional co-factors, such as kindlins, might be required for this function of talin. Here we developed a reconstituted liposome system to study In Vitro integrin ßIIß3 activation in a defined system by measurement of the binding of an activation-specific monoclonal antibody. Cryoelectron tomography images
confirmed the successful reconstitution of the integrin into liposome with approximately half of the integrins in a right side out orientation. In this In Vitro system, active fragments of talin, containing its integrin-binding PTB -like domain, were sufficient to activate highly purified integrin αIIbβ3 when both species were present at approximately equimolar concentrations. This activity of talin required that the integrin be inserted into a lipid bilayer, as the same fragments of talin failed to activate detergent-solubilized αIIbβ3 even when present at 1000 fold molar excess. Furthermore, mutations in talin predicted to disrupt membrane binding sites or that block integrin binding, inhibited its capacity to induce activation either in this purified system or in cells. Furthermore, these fragments of talin failed to activate integrin αIIbβ3 in which the β3 cytoplasmic domain had been cleaved with calpain to remove the talin binding site. Thus, we provide the first proof that talin binding alone is sufficient to activate integrin αIIbβ3 and have developed a system that will be tractable for further structural analysis of integrins activated by physiologically relevant interactions.

1275/B433
Outside-in Signal Transmission by Conformational Changes in Integrin Mac-1.
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Integrin receptors mediate bi-directional signaling between the cell and its extracellular environment. The affinity of integrins for ligand is regulated by conformational changes that occur when an integrin is activated by “inside-out” signals. Adherence of the high affinity integrin to ligand triggers “outside-in” activation of intracellular signal transduction pathways that regulate cell functions, such as proliferation, differentiation, motility, and programmed cell death. Little is known about the role of integrin conformational changes in outside-in signaling. The objective of our study was to determine the role of structural conformation in outside-in signaling mediated by the leukocyte integrin Mac-1 (αMβ2). We found that the binding of monovalent Fab fragments of a Mac-1 activating antibody was sufficient to trigger intracellular signaling in human neutrophils in the absence of ligand binding. We demonstrate using fluorescence resonance energy transfer assays that both anti-Mac-1 activating Fab fragments and ligand ICAM-1 binding shifted the conformational equilibrium of Mac-1 toward the extension of its extracellular domain and separation of its cytoplasmic tails, two hallmarks of integrin activation. These results suggest that the active Mac-1 conformer can initiate outside-in signaling in the absence of ligand binding. Our data predict a potential side effect by the ligand mimetic anti-adhesive immunotherapies targeting integrins, which block ligand binding but could produce undesirable outside-in signals by inducing conformational changes in the integrin.

1276/B434
Role for ADAP in Shear Flow-induced Platelet Mechanotransduction.
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Role for ADAP in Shear Flow-induced Platelet Mechanotransduction. A. Kasirer-Friede1, Z.M. Ruggeri2, S.J. Shattil1. 1Department of Medicine, University of California, San Diego; 2Department of Molecular and Experimental Medicine, Scripps Research Institute. In order for platelets to participate in normal hemostasis and arterial thrombosis, they must establish stable adhesions to vascular matrices at the shear stresses present in the circulation. While this process requires integrin αIIbβ3, the molecular mechanisms of αIIbβ3 signaling to cytoskeletal elements under stress (mechanotransduction) are poorly understood. Platelets adherent to immobilized fibrinogen via αIIbβ3 undergo Src-dependent tyrosine phosphorylation of multiple proteins. One such protein is ADAP (adhesion and degranulation promoting adapter protein), which binds actin-regulatory proteins SLP-76, VASP and SKAP-HOM. Here we used mice deficient in ADAP or one of its
binding partners to investigate ADAP’s role in mechanotransduction in platelets. ADAP+/− mouse platelets formed unstable rather than normal stable thrombi in response to carotid artery injury. When examined by live video microscopy, ADAP+/− platelets exposed to fibrinogen under shear flow exhibited a 45-60% decrease in stable adhesion (p<0.05), and a 55% (p< 0.05) decrease in matrix contact areas compared to ADAP+/+ platelets. Moreover, ADAP+/+ platelets, but not ADAP−/− platelets, assembled F-actin-rich structures that served as a hub for the accumulation of SLP-76 and the Rac1 exchange factor, phospho-Vav1, the latter a SLP-76 binding partner. These defects in adhesion, spreading and F-actin assembly of ADAP+/− platelets could not be rescued by co-stimulating them with a PAR4 receptor agonist or by direct αIIbβ3 activation with MnCl2, consistent with a defect in outside-in αIIbβ3 signaling. Surprisingly, ADAP−/− platelets adhered to and spread on fibrinogen normally under static conditions. No defects in spreading were observed in platelets lacking VASP or SKAP-HOM. These results establish that ADAP is a mechanotransducer of outside-in αIIbβ3 signals that promote the assembly of F-actin-rich structures in platelets to enable thrombus formation in the face of fluid shear stress.

1277/B435
Forcing Switch from Short- to Intermediate- and Long-lived States of the AlphaA Domain Generates LFA-1/ICAM-1 Catch Bonds.
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LFA-1/ICAM-1 binding mediates leukocyte functions, under mechanical forces. To elucidate how force regulates LFA-1/ICAM-1 dissociation, we used a biomembrane force probe to measure lifetimes of single ICAM-1/LFA-1 bonds. Increasing force first prolonged lifetimes (catch) then shortened lifetimes (slip) after a maximum. Bond lifetimes were single-exponentially distributed at zero-force, became triple-exponentially distributed with increasing force, and returned to single-exponentially distributed as force increased further, identifying three states with short, intermediate, and long lifetime scales. Increasing force shifted the associated fractions from the short- to intermediate- and long-lived states and shortened their lifetime scales exponentially. Steered molecular dynamics simulations revealed stepwise shift from the up to intermediate and down conformations of the alphaA domain alpha7-helix with increasing force. An antagonist molecule XVA143 that blocks the downward pulling of the alpha7-helix had no effect on the zero-force lifetimes, but suppressed the intermediate- and long-lived states to convert catch bonds to slip bonds. These results elucidate the allosteric mechanism for the integrin mechanochemistry.

1278/B436
Demonstration of Catch Bonds between an Integrin and Its Ligand.
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Binding of integrins to ligands provides anchorage and signals for the cell, making them prime candidates for mechanosensing molecules. How force regulates integrin/ligand dissociation is unclear. We used atomic force microscopy to measure the force-dependent lifetimes of single bonds between a fibronectin fragment and an integrin α5β1-Fc fusion protein or membrane α5β1. Force prolonged bond lifetimes in the 10-30 pN range, a counterintuitive behavior called catch bonds. Changing cations from Ca2+/Mg2+ to Mg2+/EGTA and to Mn2+ caused longer lifetime in the same 10-30 pN catch bond region. A truncated α5β1 construct containing the headpiece but not the legs formed longer-lived catch bonds that were not affected by cation changes at forces <30 pN. Binding of mAbs that induce the active conformation of the integrin headpiece shifted catch bonds to a lower force range. Hence, catch bonds formation appears to involve force-assisted activation of the headpiece but not integrin extension.
1279/B437
Clustering of $\alpha_5$ Integrins Determines Adhesion Strength whereas $\alpha_v\beta_3$ and Talin Enable Mechanotransduction.

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A key molecular link between cells and the extracellular matrix is the binding between fibronectin and integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$. However, the roles of these different integrins in establishing adhesion remain unclear. We tested the adhesion strength of fibronectin-integrin-cytoskeleton linkages by applying physiological nanonewton forces to fibronectin-coated magnetic beads bound to cells. We report that the clustering of fibronectin domains within 40 nm led to integrin $\alpha_5\beta_1$ recruitment, and increased the ability to sustain force by over 6-fold. This force was supported by $\alpha_5\beta_1$ integrin clusters. Importantly, we did not detect a role of either integrin $\alpha_v\beta_3$ or talin 1 or 2 in maintaining adhesion strength. Instead, these molecules enabled the connection to the cytoskeleton and reinforcement in response to an applied force. Thus, high matrix forces are primarily supported by clustered $\alpha_5\beta_1$ integrins, while less stable links to $\alpha_v\beta_3$ integrins initiate mechanotransduction, resulting in reinforcement of integrin-cytoskeleton linkages through talin-dependent bonds.

1280/B438
MCP-1 Enhances Monocyte Adhesion To VCAM-1 without Affecting VLA-4 Affinity.

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Monocyte Chemoattractant Protein-1 (MCP-1) is a chemokine which induces monocyte adhesion to the endothelium, leading to the formation of atherosclerotic plaques. MCP-1 induces monocyte arrest by enhancing adhesion of the monocyte expressed integrins VLA-4 and LFA-1 to their respective ligands VCAM-1 and ICAM-1 expressed on the endothelium. It is unclear whether MCP-1 affects both VLA-4 and LFA-1, or only one of the integrins. Furthermore, there is some debate whether chemokines enhance leukocyte adhesion through integrin affinity regulation. The aim of this work is to characterize the effect of MCP-1 on integrin mediated adhesion in THP-1 cells, a human monocytic cell line, using the Atomic Force Microscope (AFM). THP-1 cells were immobilized to AFM cantilevers coated with poly-L-lysine. AFM force-distance curves of THP-1 cells adhering to substrates functionalized with either VCAM-1 or ICAM-1 and/or MCP-1 were acquired. Detachment work, maximum detachment force, and maximum detachment distance were determined from AFM force-distance curves where THP-1 cells were pressed against substrates with ~500 pN of force for 0.1s. Membrane tethers or adhesion events exhibiting a force plateau >0.25 µm supported by only one visible rupture were also measured. Lifetime versus extraction force of VCAM-1 tethers was fitted to the Bell model to determine the intrinsic interaction lifetime ($k_{off}$). VCAM-1 tether viscosity was determined from the relationship between tether extraction force and speed. Single molecule force measurements were acquired to examine whether MCP-1 enhances VLA-4 affinity to VCAM-1. In conclusion, MCP-1 had no apparent effect on LFA-1 adhesion at short contact times as there was no apparent increase in detachment work, maximum detachment force, and distance. In contrast, VLA-4 adhesion to VCAM-1 was enhanced by MCP-1 but not by affinity regulation as demonstrated in single molecule rupture force measurements and lifetimes determined from single membrane tether measurements. Lastly, MCP-1 did not significantly affect the viscosity of VCAM-1 tethers. These results elucidate the mechanisms by which MCP-1 enhances integrin mediated monocyte adhesion.

1281/B439
Lateral Mobility of Transfected PSGL-1 on K562 Cells Enhances Adhesion by Increasing Cooperativity with $\beta_1$ Integrins.

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Leukocyte-endothelial cell adhesion is an early, critical step in inflammation. A major mechanism in activation of leukocyte adhesion is activation of integrins by multiple mechanisms, not all of which involve affinity changes. For example, β2 integrins can modulate adhesion independently of their conformational changes, through lateral mobility and rearrangement. In the current study, our objective was to determine whether lateral mobility of another adhesion molecule, PSGL-1, was also pro-adhesive, and whether this was independent of integrin activity. PSGL-1 with a truncated cytoplasmic domain (ΔC) was expressed on K562 cells to provide a cell system in which the PSGL-1 was constitutively mobile. Adhesive function under flow conditions was then compared to K562 cells expressing wild type (WT) PSGL-1. Similar distribution patterns of PSGL-1 on the two transfectants were confirmed by confocal microscopy, and greater lateral mobility of ΔC compared to WT was confirmed by Single Particle Tracking (SPT). We found that the effect of PSGL-1 mobility was not the same on purified ligand as on a HAEC monolayer. WT PSGL-1 was more than adherent than ΔC on the P-selectin substrate (5.93±0.79 cells/minute vs. 2.63±0.24, p<0.001), but, in contrast, ΔC was more adherent on the HAEC substrate (4.27±0.3 vs. 8.18±0.70, p<0.01). Fixation did not change WT PSGL-1 total adhesion on HAEC. In ΔC expressing cells, however, loss of diffusion following fixation significantly reduced total adhesion (8.50±0.56 vs. 3.72±0.60, p<0.01). Interestingly, function-blocking anti-β1 integrin antibody blocked ΔC PSGL-1, but not WT PSGL-1, adhesion on HAEC (9.04±0.84 vs. 3.22±0.39, p<0.01). Our model to explain these findings is that, although ΔC PSGL-1 diffusion may increase kinetics of PSGL-1/P-selectin encounters, this effect is not sufficient to increase PSGL-1 adhesion on substrates without β1 integrin ligands. On HAEC cells, however, PSGL-1 mobility increases adhesion by permitting PSGL-1 to rearrange in a way that enhances cooperativity with β1 integrins.

1282/B440
Analysis of Single Integrin Behavior in Living Cells.
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Integrins are trans-plasma membrane receptors that mediate linkages between the extracellular matrix (ECM) and the actin cytoskeleton. In migrating cells, integrins dynamically cluster together into focal adhesions (FAs). Integrin binding to ECM involves induced conformational changes that increase the affinity of the extracellular domains to ECM ligands, while indirect protein-protein interactions in FAs mediate cytoskeletal linkages. How the movement of individual integrin molecules in the plasma membrane relates to integrin activation, ECM or cytoskeletal binding, and FA formation is not known. To address these questions, we analyzed the dynamics of single integrin molecules in migrating U2OS osteosarcoma cells. Cells expressing integrin αv or a farnesylated peptide (CAAX) fused to the photoconvertible fluorescent protein, tdEos, were analyzed by single particle tracking photoactivation localization microscopy (SPT-PALM). We imaged a high density of single molecules of photoconverted tdEos by total internal reflection fluorescence microscopy (TIRFM) and tracked their trajectories using a globally-optimal SPT algorithm. Analysis of CAAX trajectories revealed that a majority of molecules (84%) exhibited Brownian diffusive behavior with 7% showing confined diffusion (D = 2.5-3x10^-13 m^2/s). In contrast, a majority of integrin αv trajectories exhibited confined diffusive behavior (74% confined, 21% Brownian; D = 2.5x10^-14 m^2/s). Image segmentation allowed classification of trajectories inside and outside FAs. Surprisingly, this showed that the proportion of confined or Brownian...
diffusive behavior was independent of whether molecules were inside or outside FAs. Analysis of integrin αv molecules specifically within FA showed that treatment with Mn^{2+} to induce integrin activation reduced the confinement radius of diffusion, whereas disruption of the actin cytoskeleton with latrunculin-A significantly increased the confinement radius. These results indicate that integrins can be immobilized without clustering, but can also remain mobile while clustered within FAs. In addition, both activation and cytoskeletal connection contribute to reduced integrin mobility within FAs.

1283/B441
Distinct Domains in the β5 Integrin Cytoplasmic Tail Are Required For Receptor Adhesive Function and Polarity.
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Robust adhesion to adjacent intact photoreceptor outer segments and diurnal phagocytosis of shed photoreceptor outer segment fragments are two functions of the retinal pigment epithelium (RPE) that are essential for vision. Both depend on the integrin receptor αvβ5, which localizes to the apical surface of RPE cells in the eye facing photoreceptors and of RPE cells in culture facing the tissue culture medium. In contrast to αvβ5, numerous other integrin receptors including the related αvβ3 localize to the basolateral surface of RPE cells. We thus hypothesized that the β5 subunit cytoplasmic tail contains trafficking motifs that promote the specific apical polarity of αvβ5 heterodimers. To identify such motifs, we generated expression plasmids encoding full-length β5 fused to GFP as well as β5-GFP mutants harboring deletions or point mutations within the carboxyterminal 40 residues. In non-polar cells, all β5-GFP mutants reached the cell surface as αv heterodimers like full-length β5-GFP. However, deleting β5 regions 40-30, 30-20, or 20-10 amino acid residues from the C-terminus or mutating single lysines or tyrosines diminished αvβ5-GFP’s ability to function in adhesion. Full-length β5-GFP localized apically in RPE and promoted phagocytosis. Notably, β5-GFP localized basally in polarized MDCK epithelial cells indicating that apical polarity of αvβ5 is RPE-specific. Testing polarity of the β5-GFP mutants in MDCK cells revealed that lack of regions 30-20 or 20-10 reversed the polarity of β5-GFP. In contrast, region 40-30 localized basally like full-length β5-GFP. These results suggest that distinct regions of the β5 integrin cytoplasmic tail are required for adhesive function or for polarized distribution of αvβ5 receptors. Currently, we are testing which of the domains is required for the phagocytic function of αvβ5. Initial assays suggest that expression of αvβ5-GFP in cells that normally lack β5 and do not phagocytose is sufficient to promote binding of outer segment particles, a prerequisite for phagocytic engulfment. Our ongoing experiments seek to identify cytoplasmic proteins that specifically interact with the individual regions of the β5 tail and that are essential for αvβ5 receptor activities.

1284/B442
Vinculin Activators Increase Integrin Function and Melanoma Chemosensitivity.
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Current therapies aimed at targeting cell adhesion are directed towards modulating the interaction of integrins, the major cell surface adhesion receptors, with the extracellular matrix. However, a complete loss of integrin function can lead to mechanism-based toxicities. An attractive alternative approach is to target integrin associated proteins. Vinculin, an actin binding protein recruited to integrin β1 cytoplasmic tail, regulates the formation and lifetime of integrin-containing protein complexes. Here we have investigated the effect that a vinculin activating peptide (VAP) has on integrin-mediated adhesion and diseased states. We found that the VAP increases integrin-mediated signaling and adhesion to fibronectin and collagen in an integrin- and vinculin-dependent manner. We have investigated the mechanism for these effects and have found that VAP cannot increase adhesion in the presence of a mutant version of vinculin harboring an amino
acid substitution in a hydrophobic pocket in the vinculin head. Furthermore, we have explored the potential therapeutic efficacy of VAP and found that it increases integrin function in M21 melanomas and increases their sensitivity to cancer chemotherapeutics. Taken together these findings show that integrin function can be manipulated by targeting proteins that associate with its cytoplasmic domain, and that these targets may be employed as effective adjuvants to melanoma chemotherapy. These findings lay the foundation for the development of a new class of therapeutics that target cell adhesion.

1285/B443
Functional Characterization of Three RGD Mutants of the Recombinant Mojastin Disintegrin.
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Cells in a multicellular organism interact with the extracellular matrix through the binding of adhesion molecules and cell surface receptors, such as integrins. Integrins can also transfer a signal that induces a cell to migrate, proliferate, or undergo apoptosis. Disintegrins can bind and alter integrin signaling. Three different mojastin disintegrin mutants were produced with single and double mutations at the two amino acids c-terminal to the RGD motif. To create a three-dimensional model, MODELLER was used which possessed over 91% of its residues in favorable regions. These mutations resulted in an altered orientation of their RGD loop and C-termini. Thus, we hypothesized that mojastin mutants may have different binding affinities to receptors due to variations in the stereochemistry of the RGD loop and the C-termini. r-mojastin proteins were produced and purified as GST-fusion proteins. GST-mojastin WN and mutant GST-mojastin NN inhibited ADP-induced platelet aggregation in platelet rich plasma. The GST-mojastin WN was more potent with an IC50 of 8.95 mg/ml, while the GST-mojastin NN had an IC50 of 16 mg/ml. The GST-only protein and the mutant GST-mojastin DM did not inhibit platelet aggregation. All three GST-mojastin proteins inhibited T24 cell adhesion to fibronectin. The GST-mojastin NN was more potent (IC50 of 0.38 mg/ml), followed by the GST-mojastin DM (IC50 of 1.10 mg/ml), while the r-mojastin WN had an IC50 of 6.79 mg/ml. The GST-mojastin WN was more potent at inhibiting the binding of SK-Mel-28 cells to fibronectin with an IC50 of 0.36 mg/ml, followed by the GST-mojastin NN (IC50 of 0.90 mg/ml), and the GST-mojastin DM (IC50 of 1.65 mg/ml). None of the r-mojastin proteins inhibited wound healing of T24 cells. The GST-only protein failed to inhibit T24 and SK-Mel-28 binding to fibronectin and wound healing. We are currently characterizing integrin expression on these cell lines as the first step in determining which integrin receptors bind to r-mojastin mutants. This research was supported by NIH/SCORE #5 S06 GM0081079, and HHMI Science Education Grant# 52006312.

1286/B444
Analysis of Subtype Specific Integrin Cross-Talk Using Integrin Binding Peptide Conjugated Chitosan Membranes.
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Peptide-chitosan membranes promote peptide-type specific biological activity and are useful as a biomaterial for use in tissue engineering. Previously, we conjugated a syndecan binding peptide AG73 (RKRLQVQLSIRT, mouse laminin α1 chain) and an integrin α2β1 binding peptide EF1zz (ATLQLQEGRLHFXDLGKGR, X: Nle, mouse laminin α1 chain) with various ratios on a chitosan membrane. The cell attachment and cell morphology were dependent on the ratios of AG73 and EF1zz on a chitosan membrane. When AG73 and EF1zz were conjugated on a chitosan membrane with 1:9 molar ratio, the mixed-peptide chitosan membrane promoted the strong cell attachment and neurite outgrowth. Here, we focus on three integrin binding peptides, FIB1 (YAVTGRGDSPAS, human fibronectin), EF1zz, and 531 (GEFYFDRLKGDKY, human collagen α1 (IV)) that bind to αvβ3, α2β1, and α3β1 integrin, respectively. We mixed these peptides and
examined their cell attachment and cell spreading activities using human dermal fibroblasts to analyze the cross-talk between integrin subtypes. The FIB1- and EF1zz-, and 531-chitosan membranes promoted cell attachment and spreading with peptide specific morphology. When we mixed EF1zz and 531 with various ratios onto the chitosan, cell attachment and spreading activity was significantly decreased. In contrast, FIB1:EF1zz and FIB1:531 mixture did not indicate any difference. These results suggest that β1 integrin subtypes may compete or compensate each other whereas no effect on different β subunit integrin subtypes. Next, we examined neurite outgrowth activity of the peptide-chitosan membranes using PC12 rat pheochromocytoma cells. The FIB1-chitosan membrane promoted neurite outgrowth, but the EF1zz- and 531-chitosan membrane did not. The FIB1:EF1zz- and FIB1:531-chitosan membranes showed neurite outgrowth activity depending on the FIB1 amount. These results suggest that β1 integrin is not involved in neurite outgrowth activity. We conclude that the mixed peptide-chitosan membrane method has a potential to apply for analysis of biological functions among different receptors.

1287/B445
Fine Mapping of Fibrinogen Central Domain Responsible for the Binding to αXβ2 and αMβ2 Integrins.
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Fibrinogen is a major blood plasma protein which is converted into fibrin to prevent blood leaks from blood vessels. Besides blood clotting, fibrinogen is involved in additional functions including platelet aggregation, leukocytes adhesion, and stimulation of cytokine release in leukocytes. Some of these functions are mediated by the interaction of fibrinogen and β2 integrin such as αMβ2 and αXβ2. To understand the nature of fibrinogen and integrin interaction, we investigated the region of fibrinogen molecule responsible for the binding to αMβ2 and αXβ2 integrin. Previously we found that αXβ2 recognized not only the distal domain but also the central domain of fibrinogen. As a next step, we further analyzed the binding motifs of central domain of fibrinogen by using solid phase and surface plasmon resonance analysis with αM and αX I domains. To determine the responsible chain of fibrinogen for integrin binding, each chain of fibrinogen central domain were expressed and purified from bacteria for the test of I domain binding. It was shown that the β chain of the central domain of fibrinogen bound to αM and αX I-domains in a dose-dependent manner while α and γ chain did not. Several β chain specific peptides were synthesized and tested for the binding activities. As a result, B2 peptide with a sequence (TQKKVERKAPDA) was found to bind αM and αX I-domains. In addition, the mutation of E to a in this region significantly lowered the binding level of fibrinogen to the I-domains. Taken together, these results indicate that B2 peptide region of fibrinogen β chain was the critical binding site for αMβ2 and αXβ2 integrin.

Gap Junctions (1288 – 1301)

1288/B446
Dipyridamole Increases the Gap Junction Coupling of Bovine GM-7373 Aortic Endothelial Cells by a cAMP/PKA Dependent Phosphorylation Mechanism.
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Gap junction coupling in endothelial cells are involved in the regulation of vasomotility and vasopressure. They may therefore offer an interesting target in pharmacological management of vascular impairments. We used the bovine aortic endothelial cell line GM-7373 to analyse whether dipyridamole could affect gap junction coupling of endothelial cells. Scrape loading/dye transfer technique revealed that a cell treatment with dipyridamole (1-100 µM) increased the gap junction coupling in a concentration dependent manner. In further experiments we found also that similar to dipyridamole, 8-Br-cAMP or forskolin increased the gap junction coupling. Additionally,
we observed that H-89 the inhibitor of PKA could antagonise the effect of dipyridamole on gap junction coupling. We propose that dipyridamole related increase of gap junction coupling in endothelial cells is mediated by a mechanism that involves cAMP/PKA dependent phosphorylation. This modulation of gap junction coupling could participate in the positive effect of dipyridamole treatment with respect to stroke prevention.

1289/B447
Increased Apoptosis by Combinational Treatment of Gap Junction Activator and Tamoxifen in Breast Cancer Cells.
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Objective: Combinational therapies of tamoxifen and different drugs are being frequently studied. In the current study, we tested the efficacy of substituted quinolines (code name = PQ1; gap junctional activator) in combination with tamoxifen in T47D cells. Methods: Colony growth assay was performed using soft agar to measure the colony growth while cell proliferation was measured by MTT assay in T47D cells. The level of Ki67, surviving and BAX was measured using confocal microscopy along with western blot analysis. APO-BrdU labeling was also examined in the induced treatment of T47D cells. Results: We observed a 55% decrease in the colony growth in the presence of combination of PQ1 and tamoxifen; while tamoxifen alone has little effects. Combination of 10 µM tamoxifen and PQ1 200 nM or 500 nM resulted in only 16% cell viability compared to controls at 48 hr in T47D cells by MTT assay. We found a significant increase in BAX protein at 1 hr in the presence of 500 nM PQ1 alone, 10 µM tamoxifen alone and combination of PQ1 and tamoxifen. A 2-fold increase was observed in active caspase 3 in the presence of combinational treatment of 10 µM tamoxifen and 200 or 500 nM PQ1. Also, flow cytometric analysis showed a 50% increase in the number of apoptotic cells in the presence of combination of tamoxifen and PQ1 compared to the control. Furthermore, the results show that combinational treatment of tamoxifen and PQ1 significantly reduces the expression of survivin in T47D cells. Conclusions: The combinational treatment of PQ1 and tamoxifen has a significant increase in BAX expression, caspase 3 activation and DNA fragmentation. Tamoxifen alone and combination with PQ1 showed a decrease in the survivin expression while PQ1 alone shows to be independent of survivin-mediated pathway. This suggests that an increase in gap junction activity can potentiate the effect of tamoxifen. The present study demonstrates for the first time that combinational treatment of tamoxifen and PQ1 (gap junctional activator) can be used to potentiate apoptosis of T47D human breast cancer cells. Thus, gap junctional activator, PQ1, could alter either the length or dose of tamoxifen clinically used for breast cancer patients.

1290/B448
Drosophila Innexins Ogre and Inx2 Co-Localize and Interact to Form Heteromeric Gap Junctions.
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Gap junctions are clusters of intercellular channels that regulate the exchange of ions and small signalling molecules between neighbouring cells. The structural components of these channels are the products of two gene families: connexins are exclusive to chordates while innexins/pannexins are found in both prechordates and chordates. The genome of Drosophila melanogaster contains eight innexin (Inx) genes. One of these, optic ganglion reduced (ogre), was originally identified as a gene essential for postembryonic neurogenesis. The CNS, in particular the optic lobes, are smaller in ogre mutants than in wild-type because of defects in proliferation or survival of neuroblasts (Lipshitz & Kankel, Dev Biol. 108: 56-77, 1985). We have found that Ogre fails to form gap junctions independently. The objective of this study was to identify other innexins with which it functionally interacts. Using innexin-specific antibodies, we have demonstrated, by immunofluorescence microscopy, that Ogre co-localizes with innexin2 (Inx2) in neuroblasts and glial cells of the larval CNS. Anti-Inx2 antibodies co-precipitate Inx2 and Ogre from membrane extracts of Drosophila tissues, indicating that these proteins are part of the
same molecular complex. Dual voltage clamp electrophysiology of paired *Xenopus* oocytes co-injected with *ogre* and *inx2* RNAs demonstrates that these innexins form functional heteromeric channels, with voltage properties distinct from *Inx2* homotypic channels. These data suggest that *Ogre* acts with *Inx2* to regulate signalling between neuroblasts and/or surrounding glial cells during postembryonic development of the *Drosophila* CNS.

1291/B449

**Analysis of Mutations in Connexin26 Associated with Syndromic Deafness.**

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Mutations found in connexins play a large role in both syndromic deafness and non-syndromic deafness. A majority of non-syndromic deafness is caused by mutations in Connexin26 (*Cx26*), which can also cause syndromic hearing loss associated with skin disease. Non-syndromic hearing loss can be caused by severely truncated *Cx26* proteins that lack all function, but these same mutations do not cause skin disorders. This suggests that the skin diseases associated with syndromic deafness are not the result of a simple loss of function, but by a novel gain of function that manifests in the resulting disorder. Some mutations resulting in the syndromic disorder Keratitis-Ichthyosis Deafness (KID) syndrome displayed increased hemichannel activity. In order to further evaluate this, we compared a previously characterized KID mutation, N14K, with a second KID mutation at the same location in the protein N14Y. By testing two naturally occurring mutations from the same protein residue we hoped to see what differences or similarities would result. We used dual and single whole cell voltage clamp techniques to measure the activity of both hemichannels and complete gap junction channels. The proteins were expressed In Vitro using the *Xenopus* laevis oocyte expression assay. Expression of N14Y in cells resulted in hemichannels that were non-functional compared to wild-type *Cx26* proteins and that had a current-voltage relationship that was nearly identical to H2O injected negative control cells. Cells expressing N14K mutated proteins resulted in robust hemichannel currents that were several times larger than wild-type currents. In a paired cell system, cells injected with N14Y protein failed to yield functional gap junction channels, while N14K formed channels with altered voltage gating. These results suggest that the gain of function mechanisms causing syndromic deafness may vary between mutations in the same gene, and that mutation of a single residue into different amino acids may have disparate effects on homeostasis.

1292/B450

**Interactions of Viral and Cellular Innexins in Paired Xenopus Oocytes.**

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Innexins are the channel-forming proteins of insect gap junctions, analogous in function to the mammalian connexins. Innexin homologues are present in the genomes of ichnoviruses, polydnaviruses that are carried by parasitic wasps of the Ichneumonidae family (Kroemer & Webb, Ann. Rev. Entomol., 49:431-56, 2004). The virus is transferred, along with the wasp egg, to host lepidopteran larvae; virus-encoded proteins are believed to suppress the immune response, or interfere with development, of the host thereby facilitating successful parasitization. We hypothesize that viral innexins (vinnexins) aid parasite survival by disrupting the function of host gap junctions, for example in haemocytes, the insect immune cells. To begin to test this hypothesis, we have expressed vinnexins and host innexins, alone or in combination, in paired *Xenopus* oocytes and assessed the formation of gap junctions by dual voltage clamp electrophysiology. Previously, we reported that two of the four vinnexin (Vnx) genes of *Campoletis sonorensis* ichnovirus, VnxD and VnxG, form functional intercellular channels in this In Vitro system (Turnbull et al., Curr. Biol., 15: R491-492, 2005). Here we show that VnxQ1 and VnxQ2, likewise, form homotypic channels and, moreover, that all four Vnxs are capable of interacting with innexin2 (*Inx2*) of the lepidopteran, *Spodoptera frugiperda*. *Inx2*, expressed alone, forms intercellular channels with high average conductance. Channel conductance is significantly
reduced when VnxD or VnxQ1 is co-expressed with Inx2, suggesting that these vinnexins form heteromeric complexes with Inx2. All four Vnxs form heterotypic channels with Inx2 when expressed in neighbouring cells of a pair. These data are consistent with a role for vinnexins in altering gap-junction communication in vivo.

1293/B451
Internalized Gap Junctions are Degraded by Autophagy.
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Direct cell-cell communication mediated by plasma membrane (PM)-spanning gap junction (GJ) channels is vital to all aspects of cellular life. Obviously, GJ mediated intercellular communication (GJIC) requires precise regulation. However, cells not only need to be able to precisely modulate GJIC, but also to internalize GJs (physically uncouple from each other) if they need to become migratory (development, tissue differentiation, wound healing, etc.), or need to uncouple for other reasons (mitosis, apoptosis, extravasation etc.). We discovered that cells internalize their GJs in response to various endo- and exogenous stimuli in a clathrin-mediated process. Interestingly, internalization yields double membrane GJ vesicles, termed annular gap junctions (AGJs), with lumen and inner membrane being derived from the cytoplasm and PM of the neighboring cell and the outer membrane from PM of the internalizing cell. Besides its severe inhibitory impact on GJIC and physical cell-cell coupling, endocytosis of such large structures from the PM represents a fascinating cell biological problem. As AGJs accumulate in the cytoplasm they need to be processed and potentially degraded. Autophagy, when discovered was believed to be a degradation strategy that cells, especially under starvulation use to restore essential molecular nutrients. However, more recent studies show that autophagy is a common cellular degradation pathway that is used widely to degrade oligomeric protein complexes including organelles, disease-linked protein aggregates, mitotic midbody rings, etc. The GTPase Rab5, ubiquitination, and the aggregate-mediator protein p62/sequestosome1 have been linked to autophagosomal degradation, and we show that they interact with internalized GJ vesicles. Additionally, using a set of chemical compounds that functionally inhibit cellular endo/lysosomal, and autophagosomal protein degradation pathways, RNAi-mediated protein depletion, immuno-colocalization and ultrastructural analysis we demonstrate that autophagy is the normal degradation pathway that cells use to dispose off internalized GJ complexes.

1294/B452
Vascular Gap Junction Cx37 Uncoupling by Tumor Necrosis Factor is Associated with Changes in Cx37 Interaction with ZO-1 but not actin and N-cadherin.
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Regulation of gap junctional intercellular communication plays a very important role in many physiological and pathophysiological processes. Despite significant knowledge of the role of endothelial cells during inflammation, the function of specific endothelial connexins during inflammation is not well understood. Our hypothesis is that tumor necrosis factor (TNF) will decrease gap junction dependent cell-to-cell communication of vascular connexin by disturbing connexin-cytoskeleton interactions. Transformed HeLa cells expressing vascular connexin 37, 40 or 43 (gift from Dr. Klaus Willecke) were used in these experiments. HeLa cells were treated with TNF (20 ng/ml) for up to 2 h. In dye-transfer experiments, carboxyfluorescein (HeLaCx40 and HeLaCx43) or Alexa Fluor-480 (HeLaCx37) was injected into one cell for 10 s and cell transfer allowed to proceed for 10 m and the number of labeled cells counted. Cell lysates were prepared and ultracentrifuged. ZO-1, N-cadherin, actin, Cx37, 40 and 43 were detected by Western blot. Cx37 was also immunoprecipitated (IP) overnight and precipitated. After 1 hour, TNF treatment resulted in near total loss of dye-coupling in HeLaCx37 and HeLaCx43 (p<0.02, n=14-16) and remained constant up to 2 hours. Dye coupling in HeLaCx40 cells remained unchanged after 1 hour and decreased after 2 hours (p<0.05, n=10). Western blots indicated that TNF treatment did not affect detergent solubility of Cx40 and 43. However, TNF caused a significant increase in detergent solubility of Cx37. ZO-1 was co-IP with Cx37 only after TNF treatment, suggesting that
TNF induces a ZO-1 and Cx37 interaction. Actin was co-IP with Cx37 but TNF did not affect this association. N-cadherin was not co-IP with Cx37. Immunofluorescence double labeling for Cx37/ZO-1 and Cx37/actin confirm the co-IP experiments. TNF reduces gap junction coupling of Cx37, 40 and 43 when expressed alone in epithelial cells. The loss of Cx37 function maybe due to the loss of detergent resistance, suggesting dissociation of Cx37 plaques. TNF mediates Cx37 interaction with ZO-1 but not actin. N-cadherin does not interact with Cx37. TNF may affect Cx37/ZO-1 interaction resulting in reduced dye coupling.

1295/B453
Gap Junction Turnover is Achieved by The Internalization of Small Endocytic Double-Membrane Vesicles That Coincides With Channel Accrual.
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Double-membrane spanning gap junction (GJ) channels typically cluster into two-dimensional arrays, termed plaques, to provide direct cell-to-cell communication. GJ plaques can be composed of a few to many thousands of channels, and range from tens of nanometers to micrometers in diameter. GJ plaques often contain circular, channel-free domains (app. 0.05-0.5 μm in diameter) that were identified more than 30 years ago and were termed nonjunctonal membrane domains or particle-free zones. Here we show, by expressing the GJ protein connexin43 (Cx43) tagged with GFP, or tagged with the novel fluorescent protein Dendra2 that can be photoconverted permanently from green to red fluorescence, that nonjunctonal membrane domains appear to be generated and are left behind by the internalization of small GJ channel clusters that bud over time from central plaque areas. Channel clusters are internalized within seconds and form endocytic double-membrane GJ vesicles with an apparent diameter of approximately 0.18-0.27 μm. GJ vesicles moved away from the bud sites deeper into the cytoplasm and were degraded by lysosomal pathways. Surprisingly, nonjunctonal membrane domains were not repopulated by surrounding channels, rather they were mobile, could fuse with each other, and were expelled at plaque edges. Quantification of internalized, photoconverted Cx43 vesicles indicated a GJ plaque half-life of 2.6 hours that falls within the half-life of 1-5 hours reported for GJs. Together with previous publications that revealed a continuous accrual of newly synthesized channels along plaque edges and simultaneous removal of channels from central plaque areas, our observations suggest how the known dynamic channel replenishment of functional GJ plaques can be achieved. Also, photoconversion of Cx43-plaques confirmed accrual of newly synthesized GJ channels along the outer periphery of plaques as has been reported previously by Gaietta et al. (2002) and Lauf et al. (2002), and not throughout plaques as has been proposed recently to supposedly occur with the surprisingly short half-life of only 2.8 minutes by Shaw et al. (2007). (Published as Falk et al., 2009, Mol. Biol. Cell 20:3342-3352)

1296/B454
Increased Astroglial Hemichannel Activity Induced by β-Amyloid Is Associated with Neuronal Death in Brain Slices.
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Alzheimer disease (AD) is an age-dependent neurodegenerative disorder characterized clinically by progressive loss of cognitive function and memory. The extracellular deposition of amyloid-β peptide (Aβ) in senile plaques and gliosis are common features of this disease. Nevertheless, the mechanism of action of Aβ is not completely understood. As inflammatory conditions increase the opening probability of astroglial hemichannels (HC) formed by connexin43 (Cx43), we investigated whether Aβ25-35 could affect astroglial HC activity and neuronal viability in acute brain slices. For that purpose, acute hippocampal slices obtained from GFAP-eGFP transgenic mice were treated for 3 h with 10 μM Aβ25-35 alone or in the presence of the cannabinoids: WIN 55,212-2 (WIN, 5 μM), 2-arachinonylglycerol (2-AG, 5 μM) or methanandamine (Meth, 5 μM).
Then, astroglial HC activity and neuronal death were evaluated by ethidium (Etd) uptake and Fluoro-Jade C positive cells, respectively. Aβ25-35 increased Etd uptake in astrocytes (350%) and neurons (450%) compared to control slices that was completely inhibited by connexin and pannexin HC blockers, respectively. Moreover, Aβ25-35 did not increase astroglial HC activity in slices from connexin 43 knock-out mice while no neuronal death was observed. In addition, Aβ25-35 induced neuronal death of pyramidal neurons, which was prevented with connexin and pannexin HC blockers. All Aβ25-35 effects on astroglial HC activity and neuronal death were blocked with the co-incubation of cannabinoids: WIN, 2-AG or Meth. Thus, connexin and pannexin HC in astrocytes and neurons, respectively, may be novel therapeutic targets to reduce neuronal death during AD.

1297/B455
Connexin43 Domains Involved in Gap Junction Internalization.
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Gap Junctions (GJs) mediate direct transfer of signaling molecules from cell to cell by forming clusters (termed plaques) of hydrophilic membrane channels. Connexins (Cxs) are the transmembrane proteins that form these channels. Complete double-membrane spanning GJ channels are formed when two hexameric hemi-channels (termed connexons) dock in the extracellular space. Docked GJ channels cannot be separated into hemi-channels under physiological conditions, and we have shown that GJs are internalized as a whole in a clathrin-mediated endocytic process to modulate GJ mediated intercellular communication and physical cell-cell coupling (Piehl et al., 2007; Gumpert et al., 2008; Baker et al., 2008; Gilleron et al., 2008; reviewed in Pauly and Drubin, 2007). Clathrin normally does not bind directly to its cargo. Instead, it requires adaptor proteins to link the cargo to clathrin molecules. We found several potential binding sites for (I) the classical clathrin-adaptor protein complex AP-2 (binding motif YXXΦ), and (II) the alternative clathrin-adaptor Dab2 (binding motif N PXY) in the cytoplasmic regulatory C-terminal domain of Cx43, both shown to be involved in GJ internalization. To characterize the Cx43 internalization signal, we mutated the potential binding sites of both adaptor proteins by site-specific mutagenesis. We have now generated 9 different Cx43 mutants including 4 tyrosine point mutations (Y to H), 3 small (3-7 amino acids) and 2 large (36 and 128 amino acid) deletion mutants, and phenotypic analyses of these mutants in cell culture is currently carried out. We expect that appropriate mutants will increase GJ half-life and size, and interfere with GJ internalization. We anticipate that these analyses will elucidate how, mechanistically, clathrin can internalize such large plasma membrane structures.

1298/B456
Alterations in Connexin-43 after Exposure to the DNA Damaging Drug Mitomycin-C.
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Gap junction intercellular communication (GJIC) connects the cytoplasm of adjacent cells and allows intercellular transfer of small molecules. Connexin proteins compose the channels of GJIC and can be rapidly altered in response to various cell stressors to prevent or enhance cell-cell communication which may ultimately have a role in cell survival/death. The current study explores the effects of DNA damage stress by the DNA interstrand cross-linker, mitomycin-C (MMC), on cell-cell communication via alterations in connexin-43 (Cx43). Post-confluent bovine corneal endothelial cells were serum starved for 24hrs before treatment with MMC and/or cycloheximide (CHX). Lysates were subjected to Western analysis for total Cx43 and Triton X-100 detergent fractionation was performed to determine Cx43 in insoluble vs. soluble fractions. Immunofluorescence was used to visualize changes in Cx43 localization. Functional assays for GJIC were performed and included Lucifer yellow scrape loading and FRAP. Intracellular reactive oxygen species (ROS) levels were determined using the dye, CM-H2DCFDA. The DNA cross-linker MMC altered levels of Cx43 without significant changes in intracellular ROS. Accumulation of Cx43 was observed at cell-cell junctions and in Triton X-insoluble fractions. The stability of pre-
existing Cx43 after CHX treatment was also increased with MMC. Despite the apparent changes in Cx43, there were no significant changes in GJIC as determined by scrape-load assay or FRAP. Our results demonstrate that CE cells alter protein levels and subcellular localization of Cx43 in response to DNA damage stress with MMC. Ongoing studies are focusing on the mechanisms that regulate Cx43 localization and stability changes during DNA damage. We propose that these changes allow for maintenance of GJIC as a protective response to DNA damage to continue cellular functions essential for tissue homeostasis in the cornea.

1299/B457

Connexin 43 Forms Complexes with Cyclin E and CDK2 in Vascular Smooth Muscle Cells.
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We previously demonstrated that following stimulation with the pro-atherogenic oxidized phospholipid 1-palmitoyl-2-oxovaleryl-sn-glycero-3-phosphorylcholine (POVPC) Cx43 was specifically phosphorylated at the serine (pS) 279/282 sites within vascular smooth muscle cells (VSMC) which correlated with enhanced VSMC proliferation both in Vivo and in vitro. We hypothesized this may be due to interactions with cell cycle proteins occurring specifically following post-translation modification of Cx43. We therefore investigated whether Cx43-pS279/282 site was initiated to promote interactions of cell cycle proteins involved in the G1 to S stage of cell division. As cyclin E is prominent at the cell membrane in an inactive form and Cx43 is phosphorylated at the S279/282 site at the cell membrane, we first investigated whether Cx43 and cyclin E were co-expressed in lipid rafts and found them both to localize to GM1-positive fractions. We then identified whether Cx43 and cyclin E formed complexes by co-immunoprecipitation. Both Cx43 and cyclin E could be readily detected on Cx43 and cyclin E coated beads. In addition, both complexes were shown to contain CDK2, but not retinoblastoma protein. This observation was further confirmed by mass spectrometry of each of the precipitates. Our data suggests that Cx43 promotes cell cycle progression by direct interaction with the cyclin E/CDK complexes which is possibly regulated through post-translational modifications of Cx43.

1300/B458

Overexpression of Cx46 Causes Proteosome-Dependent Degradation of Cx43 in Lens.
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Objective: Vertebrate lens primarily contains three connexin isoforms connexin 43(Cx43), connexin 46(Cx46) and connexin 50(Cx50). The outer layer of epithelial cells in lens mainly express Cx43 and Cx50 whereas the inner layer of differentiating fiber cells and mature fiber cells degrade Cx43 and preferentially express Cx46. The purpose of this study is to investigate if overexpression of Cx46 mediates the degradation of Cx43 by the proteosomal pathway in lens. Methods: The rat full length Cx46 cDNA was cloned into PEGFP-N3 vector and rabbit lens NN1003A epithelial cells were stably or transiently transfected with the construct. The level of Cx43 protein was determined by western blot and the level of Cx43 mRNA was determined by reverse transcriptase (RT)-PCR in transfected cells. The cells overexpressing Cx46 protein were treated with 100uM protease inhibitor ALLN (N-Acetyl-Leu-Leu-Nle-CHO) to assess effects on Cx43 degradation. The increased ubiquitination of Cx43 in NN1003A cells overexpressing Cx46-GFP protein was investigated by immunoprecipitation studies where Cx43 was pulled down, from whole cell lysate, by anti-Cx43 antibody followed by immunoblot with anti-ubiquitin antibody. Results: Overexpression of Cx46 protein caused a decrease in endogenous Cx43 protein level both in stably or transiently transfected rabbit lens NN1003A epithelial cells. In transiently transfected cells the level of Cx43 protein was reduced at 36 h and 48 h after Cx46-GFP
construct transfection. Cx43 mRNA level was not altered indicating reduction in Cx43 protein level is not due to inhibition of transcription. Treatment with protease inhibitor ALLN showed no change in Cx43 protein level even in NN1003A cells overexpressing Cx46 protein. Immunoprecipitation study showed increase in ubiquitin conjugation of Cx43 in cells that overexpress Cx46 as compared to control. Conclusion: Our study demonstrates that overexpression of Cx46 initiates proteosome-dependent degradation of another gap junction protein, Cx43, in rabbit lens NN1003A epithelial cells.

1301/B459
Gap Junction Remodeling is An Important Arrhythmogenic Substrate during Development of Heart Failure.
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Heart failure is known to predispose to life-threatening ventricular tachyarrhythmias. The intercalated disc contains different junctional complexes (adhesion junction: AJ and gap junction: GJ) that enable the myocardium to function as a syncytium. The AJ play a key role in the formation and stability of GJ. We clarified the GJ connexin (Cx)43 remodeling and its potential role in the pathogenesis of arrhythmias in heart failure. We investigated changes of Cx43 in UM-X7.1 cardiomyopathic hamster hearts, and associated alterations in the electrophysiological properties using a high-resolution optical mapping system. UM-X7.1 developed left ventricular (LV) hypertrophy by ages 6~10w, and showed a moderate reduction in LV contractility at age 20w. LV mRNA and protein levels of Cx43 in UM-X7.1 were unaffected at age 10w, but significantly reduced at 20w. The Ser255-phosphorylated Cx43 expression in UM-X7.1 at age 20w was significantly greater than that in control. In UM-X7.1 at age 10w, normal LV conduction was preserved, whereas the dispersion of action potential duration (APD) was significantly increased. UM-X7.1 at age 20w showed significant reduction of cardiac space constant, decrease in conduction velocity, distortion of activation fronts and increase in APD dispersion. Programmed stimulation resulted in sustained ventricular tachycardia (VT) or fibrillation in all UM-X7.1. During development of heart failure, alterations of Cx43 expression and phosphorylation in concert with interstitial fibrosis may create serious arrhythmogenic substrate through an inhibition of cell-cell coupling. at 10w in UM-X7.1, nuclear expression of β-catenin, a constitution of AJ and enhances Cx43 transcription through an activation via a β-catenin/Tcf nuclear complex, was remarkably decreased. These results suggest alteration of β-catenin in nuclear fraction precedes the GJ remodeling, resulting in quantitative and qualitative alterations in Cx43. In conclusions, remodeling of AJ and GJ might contribute to arrhythmogenesis during development of heart failure in cardiomyopathy.

Development and Tissue-Specific Control of Gene Expression (1302 – 1320)

1302/B460
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Genomics assumes, in particular, the genome comparisons between different species and populations. The comparative analysis of polymorphism of DNA fragments, flanking by the inverted repeats of decanucleotides (RAPD), microsatellites (ISSR) and terminal sites of retrotransposon (IRAP) in genomes of domestic and closely related wild species (Bos taurus, Bison bonasus, Bison bison, Ovis aries L., Ovis nivicola borealis) was carried out. DNA regions with the high conservativeness on the length were observed. Possible connection of such conservativeness with the belonging of DNA flank fragments to purin/pirimidin tracts was discussed. It was coordinated well with the hypothesis of Lima-de-Faria about the "chromosomal fields"
about close relations between nucleotide sequences and chromosome morphology. The assuming about close connection between nanometer and micrometer genome organization was reinforced by the history of linear chromosome eucaryote appearing and participation in it of the mechanisms of retrovirus expansion, related with the centromere and telomere occurrences. With the use of DNA arrays the comparative analysis of profiles of the gene expression of two pigs organs, liver and kidney was carried out. 59 genes were revealed, which expressions were essentially above in kidney, than in liver. The possible sources of mistakes in analysis of gene expression profiles caused by "cross" hybridization of one probe with different cDNA of gene transcripts of the genes belonging to gene superfamilies, the same cDNA with different probes were discussed. The basic differences had appeared connected with the genes supervising ionic exchange, and also mechanisms of cellular division. It was corresponded well with dominating participation of kidneys in maintenance of ionic balance in blood and also with lowered activity of cytokinesis in a liver (polyploidy of hepatocytes). It was demonstrated the possibilities to use of the short DNA fragments for in-depth studies of "chromosome phenotype", genetic-biochemical mechanisms of cellular and tissue phenotype formation.

1303/B461
Divergent Roles of RelA and c-Rel in Establishing Chromosomal Loops upon Activation of the Igκ Gene.
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Precise regulation of eukaryotic gene expression requires interactions between distal cis-acting regulatory sequences with the looping out of the intervening DNA, but how trans-acting regulatory proteins work to establish and maintain DNA loops during gene activation remains largely unexplored. LPS-induced transcription of the mouse Igκ gene in B lymphocytes utilizes three distal enhancers (Ei, E3’, and Ed) and requires the transcription factor NF-κB, whose family members include RelA and c-Rel. To determine step-wise many of the requirements and events that lead to the establishment of the fully active state of the Igκ locus, we have utilized chromosome conformation capture (3C) and ChIP-3C technology along with inducible biological systems, chemical inhibitors, dominant negative and knock-down or knockout of NF-κB family members. We find that prior to transcriptional induction, rearranged V gene promoters are associated with either the enhancer Ei or E3’ (but not with both nor with Ed), forming the "poised" conformation, but that pair-wise interactions between the three enhancers occur only after LPS-activation and the transient and essential recruitment of RelA. ChIP-3C experiments reveal that these enhancer complexes are in close proximity to RNA polymerase II, providing evidence that transcription factories are the molecular ties for the bases of these DNA loops. These processes are actin-filament-dependent but independent of new protein synthesis, transcription or c-Rel. We have thus identified both essential and non-essential events that establish higher-order chromatin reorganization during Igκ gene activation. This investigation was supported by Grants GM29935 and AI067906 from NIH and Grant I-823 from the Robert A. Welch Foundation to WTG, and by Grants HL067256 and HL61897 from NIH to LST.

1304/B462
Passive Stretch Activates p38 MAPK and Prevents Muscle Atrophy in Tenotomized Rat Soleus.
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Muscle plasticity is highly influenced by mechanical cues. In response to muscle unloading or to postural changes causing muscles to work over a shortened range, the muscle undergoes atrophy. This unfavorable adaptation results in contractile weakness, structural weakness, loss of endurance and reduced range of motion. Least understood among these is the loss of range of motion. Passive stretch therapy is traditionally used to combat this lost range of motion. Relatively little is known about the mechanism by which muscle translates stretch into molecular signaling that prevents atrophy. The objective of this study was to examine stretch activation of p38
mitogen-activated protein kinase (p38), and its downstream effects as a candidate pathway for atrophy prevention signaling in an In Vivo model of muscle unloading and shortening atrophy induced by tenotomy. The soleus distal tendon was severed, and the unloaded soleus shortened. By 7 days, the soleus fibers exhibit lightly staining “cores” centrally, when reacted histochemically for actomyosin ATPase activity. The central core lesions (CCLs) represent massive breakdown of contractile proteins. Passive stretch was applied daily (beginning 24 hours after tenotomy surgery in the reanesthetized rat) via a ligature attached to the soleus and exiting the skin. on the final day, excised solei were flash frozen within 2 min of stretch release and analyzed histochemically for lesions and biochemically for p38 activation via western blot. Tenotomy alone produced 59.2 ± 11.2% fibers with CCL lesions, whereas tenotomized soleus subjected to daily passive stretch contained significantly (p<0.05) fewer CCL lesioned fibers (15.2 ± 13.3%). Tenotomized plus stretched muscles contained significantly (p<0.01) more phospho-p38 (activated) (1.29 ± 0.25 au) than sham-operated (0.03 ± 0.02 au) and tenotomized soleus (0.06 ± 0.01 au) on day 1. Similarly, a significant increase in p38 activation was present in 7-day tenotomized soleus muscle stretched daily for 20 min (0.90 ± 0.22 au) compared to sham (0.12 ± 0.04 au) or tenotomy alone (0.08 ± 0.03 au). The findings indicate that activation of p38 MAPK pathway by passive stretch prevents CCL formation in tenotomized soleus muscle.

1305/B463
Viral Gene Transfer to Developing Mouse Salivary Glands.

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Branching morphogenesis is essential for the formation of salivary glands, kidneys, lung, pancreas, and many other organs during development, but the mechanisms of this crucial process are not adequately understood. Microarray and serial analysis of gene expression (SAGE) have been powerful approaches to identify candidate genes that could potentially regulate branching morphogenesis. However, functional validation of these genes has been severely hampered by the difficulty to genetically manipulate cells within organs. Using ex-vivo cultured embryonic mouse salivary glands as a model organ system to study branching morphogenesis, we have identified new vectors for viral gene transfer with high efficiency and cell-type specificity to developing salivary glands. We screened Adenovirus and 20 different types of Adeno-Associated Viruses (AAV) for their ability to transduce embryonic day 12 or 13 salivary glands. The panel of AAVs tested included not only different serotypes but also viral capsid mutants of AAVs. Enhanced Green Fluorescent Protein (EGFP) was engineered into each of these viruses to facilitate calculations of transduction efficiency. We identified two AAV types, self-complementary AAV2 (scAAV2) and bovine AAV (BAAV), that are highly selective at targeting expression efficiently and differentially to salivary epithelial and mesenchymal cell populations, respectively. Transduction of salivary epithelia with scAAV2 that expresses fibroblast growth factor 7 (FGF7) resulted in an enhanced rate of salivary branching morphogenesis. Our findings represent, to our knowledge, the first successful selective gene targeting to epithelial versus mesenchymal cells in an organ undergoing branching morphogenesis, and the first use of gene transfer to analyze roles of growth factors in this process of mammalian organ morphogenesis. The vectors that we have identified can also be used for gene knockdown approaches. They may also be useful for experimental manipulation of other branching organ systems. Information derived from these studies may facilitate gene therapeutic strategies involving viral gene transfer to salivary glands.

1306/B464
Extended Variable Loop in FMRP KH2 Domain: Is It Functionally Relevant in Postnatal Cerebral Cortex?
Fragile Mental Retardation Protein (FMRP) is a translational regulator displaying two KH and one RGG box RNA-binding domains. Most known interactions between RNA and FMRP are mediated by its RGG box. The second KH (KH2) domain has been involved in FMRP dimerization and interaction with kissing complex RNA structures. Length variation has been predicted to the variable loop of the KH2 domain due to alternative splicing of \textit{FMR1} gene exon 12. However, it seems that the variable loop does not contribute to FMRP dimerization or binding to RNA kissing complexes. Therefore, it remains unknown if FMRP KH2 variable loop is functionally relevant. Our in silico analyses revealed that: (i) length variation within protein-specific KH domain variable loop is infrequent; (ii) \textit{FMR1} exon 12 sequence is observed only in mammals; (iii) its encoded sequence is highly conserved within this class; and (iv) in several species exon 12 may undergo alternative splicing. We raised antibodies (3460) that specifically recognize the segment encoded by \textit{Fmr1} exon 12 in embryonic rat brain. We show that exon 12-expressing FMRP isoforms are also present in postnatal rat brain with a molecular mass of approximately 75 kDa. Western blotting analyses of frontal cortex detected relatively steady levels of FMRP 75-kDa isoform between embryonic day 19 (E19) and postnatal day 10 (P10), and decreased intensity from P13 through P21. on the other hand, FMRP overall expression was comparatively stable throughout the period between E19 and P21. Our data supports a developmentally regulated role for the extended variable loop of FMRP KH2 domain in cerebral frontal cortex. As an isoform-specific antibody, 3460 serum will be useful in investigating biological roles for FMRP KH2 domain variable loop and their modulation by alternative splicing.

**1307/B465**

**Identifying Cardiac Targets of Aryl Hydrocarbon Receptor Action.**

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The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor that regulates the transcriptional activity of genes by binding to dioxin response elements (DREs). Well characterized target genes of the AhR include members of the cytochrome P450 1a1 (Cyp1a1) and phase 2 enzymes. It is clear that the AhR plays a role of cardiovascular biology, however, the repertoire of cardiac AhR transcriptional targets remains unclear. Our objective was to identify potential cardiac targets of AhR mediated gene regulation to further our understanding of the role of the AhR in cardiovascular biology. To develop a list of DRE-containing genes, we screened genomic DNA from 27967 mouse genes (-10 kbp upstream of the transcriptional start site to 1 kbp downstream of the 3’ untranslated region) for the presence of DREs using a position weighted matrix (PWM) approach and PWMs derived from Cyp1a1-like and non-Cyp1a1-like sequences. A list of cardiac expressed genes was compiled from microarray expression data representing normal and pathologic adult heart, and various stages of cardiac development. Comparison of the two lists revealed 3625 cardiac-expressed genes with 1 or more putative DREs located within 2 kb of the transcriptional start site. Pathway analysis of these 3625 genes revealed a number of potassium channel genes, including Kcnq3, Kvna6, Kcnn2, Kcnn3, Kcnn5, and Kcnj12, some of which had DRE sequences that were conserved across species. Real time-PCR was used to determine if the putative DREs in Kcnj12 were functional. There was decreased Kcnj12 expression in mouse heart after exposure to the AhR agonist 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) suggesting that the DREs in Kcnj12 are functional. AhR activity in the sample was confirmed by examining the expression of two well characterized target genes of the AhR, the AhR repressor and Cyp1a1. This study has provided new methods of identifying possible target genes of the AhR thereby aiding in our understanding the role of the AhR in cardiovascular biology.

**1308/B466**

**Structural and Genomic Profiling of the \textit{Drosophila} Cardiac Tube.**
Drosophila melanogaster possess a simple linear heart tube which is an efficient In Vivo system for studying basic developmental and physiological processes as well as for investigating potentially conserved pathogenic mechanisms of genetically inherited cardiac disorders. Human cardiomyopathies commonly arise from cytoarchitectural mutations. Here, we further characterized morphological and ultrastructural consequences of altered myosin function on the Drosophila heart and began to analyze the effects of the mutations on gene expression. Fluorescent microscopy revealed D45 flies, expressing myosin with depressed ATPase and In Vitro sliding properties, show cardiac dilation with normal myofibrillar organization. However, Mhc5 fly hearts, expressing myosin with enhanced molecular properties show centrally located restricted regions, loss of contractile material and myofibrillar disarray. Electron microscopy revealed perturbed sarcomeric organization of the cardiomyocytes in both mutants. Mitochondria appeared swollen with increased matrix volume and membranic rupture resulting in vacuolization. These cardiac phenotypes bear similarity to those observed in human cardiomyopathies and imply the existence of conserved pathological responses to altered myosin motor function. To further substantiate the use of the Drosophila heart as a model for investigating developmental, physiological and pathological processes and to identify conserved and potentially unique molecular components, we have undertaken DNA microarray analysis of isolated Drosophila hearts to determine how specific myosin heavy chain mutations affect cardiac muscle gene expression during aging. Transcriptome profiles of D45, Mhc5 and wildtype (yw) hearts were compared at 1 and 5 weeks of age. Statistical analysis allowed identification of conserved and novel gene pathways and networks involved in cardiac function and development. We ultimately seek to use the resulting gene expression patterns to implicate genetic correlates of normal cardiac aging and those involved in the development of cardiomyopathies.

1309/B467
Regulation of Constitutive Hsp27/HSPB1 Expression in the Zebrafish (Danio rerio).
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Hsp27, a 27-kDa member of the small heat shock protein family, is constitutively expressed in skeletal and cardiac muscle tissues, and overexpression of Hsp27 promotes functional recovery of muscle tissues after ischemia. Previous authors have shown that Hsp27 is expressed at high levels in slow twitch muscle lineages under non-heat shock conditions. The present study investigated constitutive distribution and regulation of Hsp27 expression in the Zebrafish, an important and genetically accessible vertebrate model. Our data show that elevated expression of Hsp27 occurs within developing slow twitch muscle fibers, but is not limited to this fiber type, and morpholino knockdown of transcription factors regulating slow twitch muscle development failed to inhibit constitutive Hsp27 expression. Knockdown of Hsp27 expression also did not alter slow twitch muscle development. Morpholino knockdown of the heat shock inducible transcription factor, Hsf1, also did not reduce constitutive expression of Hsp27, although heat shock inducible expression was suppressed. In contrast, injection of embryos with antisense morpholinos targeting the transcription factor Hsf2 did significantly reduce constitutive expression of Hsp27. Our data indicate that constitutive expression of Hsp27 is regulated, at least in part, by HSF2.

1310/B468
Protein Expression Analysis of the [Psi+] and [psi-] Yeast Strains Using 2-D Electrophoresis.
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Previously, the differences in gene expression between [PSI+] prion and non-prion [psi-] yeast strains were studied using the technique of microarray analysis. The Sup35p encodes a translational release factor, eRF3 that functions to terminate the newly synthesized polypeptide chain. Therefore, in [PSI+], eRF3 takes on an alternate conformation leading to less efficient termination and nonsense suppression. In [psi-], on the other hand, the conformation of Sup35p is fully functional and promotes efficient termination at stop codons. The microarray results showed more than 50 genes were found to be under and over expressed in [PSI+]. The central dogma of molecular biology states that DNA->RNA->PROTEIN. We theorized that if there were differences found in the gene expression between these [PSI+] and [psi-] strains, there should also be differences in the proteins produced by these strains. To test this theory, we used 2-dimensional electrophoresis. Previous methods used to extract proteins resulted in low protein concentrations being obtained. As a result, less protein spots appeared on the gel. The previous method used zirconium beads and agitation to break open the yeast cells followed by trichloroacetic acid (TCA) precipitation. In order to obtain a better protein yield, a new protocol was utilized. The new protocol uses alkaline/SDS lysis and precipitation. This method employs heat at 90 degree Celsius in alkaline lysis buffer to break open the yeast cells followed by methanol precipitation of the proteins. The new method demonstrated higher protein yields and the number of protein spots on the gel increased. The results also showed that there were protein differences found in [PSI+] when compared to [psi-] strains. Significant protein changes found from this study will be compared with the yeast gene changes found in earlier studies to determine whether gene expression changes translate into similar protein expression changes.

1311/B469
Directing Skeletal Muscle Satellite Cells into Osteogenic Lineage by Simultaneous Silencing of MRF4 and PPARγ.
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Skeletal muscle satellite cells were regarded as myogenic precursor cells, however, recent studies have shown them as stem cells capable of differentiating into other cell lineages such as adipocytes and osteoblasts. In our previous reports, MRF4 was determined to be most upstream in the transactivation hierarchy of myogenic regulatory factors to direct to myogenic lineage and MRF4 silencing induced adipogenesis, by default, in satellite cells. The working hypothesis in this study was that if both myogenic and adipogenic master control genes were silenced, satellite cells were driven into osteogenic lineage. Satellite cells were isolated from gastrocnemius muscle of 6-week-old Wister male rats by differential centrifugation and cultured in D-MEM/F12 containing 10% FBS. for gene silencing, siRNAs were designed for MRF4 and PPARγ. Satellite cells from Passage 3 were transfected with siRNA for MRF4, PPARγ, or the combination of MRF4 and PPARγ. The resulting cell lineage was determined, using MF-20 for myocytes, Oil Red O for adipocytes, and alkaline phosphatase for osteoblasts. The relative quantity of MRF4, PPARγ, and Runx2 transcripts were measured by Real-time PCR. When MRF4 alone was silenced, lipid accumulation was detected by Oil Red O. In this case, MRF4 expression was suppressed, while PPARγ and Runx2 expression remained unchanged. When PPARγ alone was silenced, myosin heavy chain was detected by MF-20. In this case, PPARγ expression was suppressed, while MRF4 and Runx2 expression remained unchanged. When both MRF4 and PPARγ were silenced, alkaline phosphatase-positive cells were detected. In this case, MRF4 and PPARγ expression was suppressed, while Runx2 expression remained unchanged. These results suggested that the hierarchy existed in the differentiation of skeletal muscle satellite cells and the ranking was placed first for myogenesis, second for adipogenesis, and third for osteogenesis. The onset of osteogenesis was induced only when MRF4 and PPARγ expression was suppressed.

1312/B470
Analysis of Transcriptional Regulation of Cadherin7 Gene Expression in Neural Crest Cells and Developing Neural Epithelium.
In the embryo, neural crest cells migrate from the neural epithelium and contribute to the formation of the peripheral nervous system, pigment cells and craniofacial structures. This migration involves changes in expression of genes such as cadherins which are regulated by various signaling pathways. During early neural development in the trunk, cadherin7 is known to be expressed in migrating neural crest cells as well as in the lateral neural tube, and later in the floor plate, where the neurites cross to the contralateral side. Identification of regulatory regions required for cadherin7 expression in migrating neural crest and its repression in dorsal and ventral neural tube will provide a better understanding of neural crest development and the developing nervous system. Using promoter prediction tools and fluorescence reporter analysis, a region of ~450 bp upstream of the first exon was identified as the promoter region of cadherin7. This region is able to drive expression of the reporter gene throughout the neural tube, including areas where cadherin7 is not expressed at these stages, suggesting the presence of a silencer element that might be repressing the expression of cadherin7 in the dorsal and ventral neural tube. Using comparative genomic analysis and transcription factor binding site (TFBS) prediction tools, nine evolutionary conserved regions (ECRs) with conserved TFBS were identified in the intergenic and intronic region of cadherin7 gene locus. Fluorescence reporter analysis identified one of the ECRs as the enhancer/repressor region of ~600 bp that appears to regulate the expression of cadherin7 in migrating neural crest cells and the neural epithelium. Further analysis of this region by biochemical analysis will identify the specific transcription factors required for expression of cadherin7 in migrating neural crest cells and repression in dorsal and ventral neural epithelium.

1313/B471

Functional Analysis of the I12b Cis-Regulatory Element That Controls Dlx Expression in the Developing Forebrain.

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During embryonic development, the Dlx homeobox genes play essential roles in the differentiation, migration and survival of subpallial precursor cells that will give rise, amongst others, to GABAergic inhibitory interneurons in the cerebral cortex. At least four cis-acting regulatory elements (CREs) are responsible for Dlx transcriptional control in the telencephalon and diencephalon: I12b and URE2 in the Dlx1/2 bigene cluster, and, I56i and I56ii in the Dlx5/6 bigene cluster. We have demonstrated that I12b, URE2 and I56i mark different progenitor cell populations in the lateral and medial ganglionic eminences (LGE/MGE) and different subtypes of GABAergic interneurons in adult animals, suggesting that distinct Dlx expression patterns may be mediated via each individual CREs. Our previous data showed that the proneural bHLH transcriptional factor MASH1 directly regulates the expression of Dlx1/2 through its action on I12b. Furthermore, I12b is a direct target of DLX2 and requires both Dlx1 and Dlx2 expression for proper activity. To investigate the relative contributions of the I12b enhancer to overall Dlx functions during brain development, we generated mice with a targeted deletion of I12b. The initial phenotype analysis of the mutant mice indicated that deletion of the I12b sequence does not affect the survival of the animals, nor the size or morphology of the cortex and hippocampus, two regions where I12b is highly active. There was no obvious disruption of tangential neuron migration, but preliminary observations suggested a reduced cell proliferation in the mutant mice, particularly in the ventricular zone of the LGE/MGE. The impact of the loss of the I12b enhancer on Dlx gene regulation and on the development of specific populations of GABAergic interneurons will be presented. These studies will provide insight into the complex and dynamic regulation of the genetic cascades involving the Dlx gene family during forebrain development. Supported by CIHR grant (MOP14460) and a travel grant from the department of Cellular and Molecular Medicine, University of Ottawa.
1314/B472
Phosphorylation of the Neural Retina Leucine-Zipper (Nrl) by JNK1 Controls Histone Acetylation and Gene Expression.
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Nrl (neural retina leucine-zipper) is a key basic motif-leucine zipper (bZIP) transcription factor, and modulates rod photoreceptor differentiation via activating the expression of rod specific target genes. By using Co-IP/ Antibody Pool-Western blot method, we demonstrated that JNK1 is a novel interacting partner of Nrl. JNK1 directly interacted with and phosphorylated Nrl. JNK1 inactive mutant or inhibitor treatment significantly reduced the JNK1-mediated phosphorylation of Nrl. Also, Ser50 phosphorylation of Nrl by JNK1 enhanced the transcriptional activity of Nrl on the Rhodopsin promoter. Consistant with biochemical assays, in the retinal explant culture, inhibition of JNK activity reduced the Nrl activation involving reduction of target gene transcripts such as Rhodopsin and Ppp2r5c. Taken together, our results demonstrate that JNK1 activation is necessary to phosphorylate Nrl at Ser50 residue and to activate a transcriptional activity of Nrl in mouse retina development. This work was supported by the Seoul R&BD program

1315/B473
The Role of bcl2 Depends on Subcellular Localization.
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Bcl2 is the best characterized member of a large family of proteins that regulate apoptosis. While it is established that bcl2 localizes at the mitochondria and functions as an anti-apoptotic protein, the function of bcl2 at the nucleus remains unclear. We hypothesized that presence of bcl2 at the nucleus may have other function than mitochondria located bcl2. We prepared a NCI-H460 human large-cell lung carcinoma line that stably overexpressed nuclear targeting bcl2. We analyzed the cells by microarrays to determine differences in gene expression. Compared to normal NCI-H460 cells, a total of 110 genes were differentially expressed by at least two-fold, 42 upregulated and 68 downregulated. Genes chosen for further analysis were confirmed by RT-PCR. We found that Reticulon3 and ST18, which enhance apoptosis, expression was increased in nuclear targeting bcl2 overexpression cells. Genes involved in cell proliferation and anti-apoptotic function, such as MCI1, RICTOR, RFC4, MCM8, were found to be decreased in nuclear targeting bcl2 overexpressed NCI-H460 cells. Taken together, these results demonstrate the possibility that a group of genes involved in DNA repair, cell proliferation and apoptosis are regulated by nuclear bcl2. This list of genes may be useful in attempts to elucidate still unknown functions of bcl2 in lung cancer cells.

1316/B474
A Contribution of Musashi1 to the Regulation by Lin-28 in Blocking miRNA Processing.
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RNA-binding proteins are important in translational regulation, with critical roles in stem cell maintenance in planarians (e.g., bruno-like) and mammalian neural stem cells (NSCs) (e.g., Musashi1). We previously reported that Musashi1 (Msi1) contributes to the maintenance of the immature state and self-renewal activity of neural stem cells through translational repression of m-Numb, and that a molecular mechanism for selective posttranslational regulation by Msi1 according to approach for identifying Msi1-containing protein complexes. Lin-28 also was included as a part of Msi1-complex. It was reported that Lin-28, which is one of the iPSC cell inducing factors, plays a central role in blocking miRNA-mediated differentiation in stem cells and in certain cancers. To address the role of Msi1 in Lin-28-complex, Microprocessing assays were performed by let-7 family miRNA. Our results demonstrate that Msi1 can facilitate the blocking miRNA-processing step, and this Msi1’s regulation occurs coordinately with Lin-28.
1317/B475

**Combinatorial Regulation of Notch Target Genes in Drosophila.**

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The Notch signaling pathway and its downstream targets in Drosophila, the *Enhancer of split (E(spl))* genes, provide an excellent model to study combinatorial transcriptional regulation. The *E(spl)* genes are all regulated by Suppressor of Hairless (Su(H)) bound co-repressors which are released in the presence of activated Notch (ICN) and its co-activators. However, these genes show variations in their expression patterns during development. Previous results support a mechanism for *E(spl)* differential expression that involves additional transcription factors interacting with Notch to regulate specific *E(spl)* expression. To better understand the complex regulation of the *E(spl)* genes we previously analyzed the upstream regulatory regions of these genes using a phylogenetic footprinting approach and identified differential sequences that were conserved in at least twelve Drosophila species. Accordingly, we found a conserved consensus site for the transcriptional repressor, Tramtrack69 (TTK69), in the promoter of one of the *E(spl)* genes, *HLHmgamma*. We have studied the possible role of TTK69 regulation of *HLHmgamma* in S2 fly tissue culture cells and in developing larval imaginal discs. We found that TTK69 inhibits ICN induction of *HLHmgamma* luciferase reporter vectors in S2 cells. This inhibition was maintained even when the TTK69 binding site was mutated in the *HLHmgamma* promoter, suggesting that the inhibitory affect is independent of DNA binding. Ectopic expression of TTK69 in larval eye imaginal discs results in an inhibition of *HLHmgamma* expression. Taken together these results suggest an interesting role for TTK69 in the regulation of ICN induced *HLHmgamma*. *HLHmgamma* has a unique, but overlapping expression pattern with other *E(spl)* genes and it is possible that TTK69 plays a role in the co-expression patterns of multiple Notch target genes. We are currently investigating this possibility.

1318/B476

**Introduction of a Foreign Gene into Breast Cancer Cells: Significance of Differentially Expressed Transcripts.**

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Many transcripts are altered within a cell following introduction of a transfected gene sequence. It is critical to distinguish transcript changes caused by the gene of interest (GOI) from changes that result from the transfection itself (vector backbone and reagent effects). In this study, MCF7 breast cancer cells were transfected by different reagents with plasmids containing secreted alkaline phosphatase (SEAP) as a model for the GOI, or an identical plasmid that did not contain the SEAP sequence. The objectives of this study were (1) to differentiate transcripts differentially expressed as a result of the GOI from those caused by transfection reagent and/or the vector backbone, and (2) to examine the changes in differentially expressed transcripts caused by each reagent during the first 48 hours post transfection. The overlaps of transcripts differentially expressed relating to objective 1 were described (BioTechniques, Vol. 47, No. 1, July 2009, pp. 617-624). While the numbers of differentially expressed transcripts were provided, the overlaps among the three time points for each reagent were not presented in that publication. Here, we describe a new analysis to investigate the importance of the transient nature of the differential expression (objective 2). Each of the four reagents tested had transcripts differentially expressed at all three time points. Additional transcripts were differentially expressed only at 8, 24 or 48 hours; other transcripts were expressed at both 24 and 48 hours. The number of unique differentially expressed transcripts at each time point varied with reagent used. The total number of unique differentially expressed transcripts was lowest for all reagents at 8 hours (2, 7, 16, and 19), moderate at 24 hours (45, 55, 148, and 425) and highest at 48 hours (124, 341, 1173, and 1303). This analysis of the transient nature of the differentially expressed transcripts clearly demonstrates the dynamics occurring; thus, depending upon the pathways affected, the post transfection time point selected for analysis may be critical when deciphering the true biologic effects of the GOI.
Identification of Methyl-Specific DNA Binding Proteins of the Bovine Nanog and POU5F1 Promoter Regions Using Electrophoretic Mobility Shift Assay.
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Deficiencies in the success rate of somatic cell nuclear transfer are widely held to be epigenetic in nature, and arise from the limited ability of a differentiated donor cell to erase epigenetic signatures required for nuclear reprogramming. Following bovine somatic cell nuclear transfer DNA methylation signatures of two genes necessary for pluripotency and self-renewal, namely Nanog and POU5F1 (Oct-4) more closely resemble that of somatic cells rather than In Vitro fertilized embryos. A retained methylation signature following scNT likely leads to interaction with methyl binding domain proteins capable of binding to methylated promoter regions and acting to silence gene expression. Using a modified version of Electrophoretic Mobility Shift Assay (EMSA) targeted to the genes Nanog and POU4F1 (Oct-4) we identified DNA binding proteins that interact specifically with the methylated promoter regions. We observed protein binding specific to a methylated DNA template that differs from the binding proteins specific to the non-methylated DNA template. These findings indicate that the gene promoter region is acted upon by different DNA binding proteins depending on its methylation status, and that the retention of a hyper-methylation signature could lead to the premature down-regulation of the genes Nanog and POU5F1.

Evolutionary and Functional Characterization of a Novel Cold Shock Domain Protein from Indian Eri silkworm, Philosamia ricini: In-silico Sequence Analysis.
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All vertebrate Y-box proteins(YBPs) that are evolutionarily most conserved nucleic acid-binding proteins, contain a variable N-terminal domain, a Cold Shock Domain( CSD) and a C-terminal tail domain and are implicated in various cellular processes, including adaptation to low temperatures, cellular growth, nutrient stress and stationary phase. We have cloned and sequenced the first cDNA, coding for Y-box protein, a member of cold shock domain proteins, from Philosamia ricini mRNA isolated from 40C acclimated 5th Instar larvae and predicted its amino acid sequence. The cloned cDNA 795 bp codes a 265 aa YBP with a 71 aa-long CSD. The deduced amino acid sequence of the YBP gene of P. ricini showed about 80% identity to its homolog present in Bombyx mori.. The motif search with the deduced amino-acid sequences showed presence of a N-terminal domain, a CSD rich in hydrophobic residues and a C-terminal domain. P.ricini CSD shares close homology with other CSDs that have RNA binding properties and most likely also has RNA binding properties and functions as a transcription factor during protein synthesis. The multiple sequence alignment showed high homology among the CSDs themselves suggesting that they are highly conserved. The phylogenetic tree was well supported by high bootstrap values and revealed common origin of all insect CSD protein family members. Based on deduced amino acid sequence and phylogenetic analyses, we confirm that P.ricini novel protein belongs to the same CSD protein family. (Supported by BIF Grant from Department of Biotechnology, Govt of India to DKG)

Mechanism of Coordinated Strand Recognition in DNA Duplex during Eukaryotic Cell Cycle.
The extremely low level (1 per 10 billion of bases) of sister chromatid exchange errors in eukaryotes shows strong evolutionary pressure on semi-conservative replication. During this process the parent DNA strands are differentiated from each other along the whole length of the chromosome. The mechanism of spatial coordination between a large number of replication forks remains unexplained. Observations of labeling patterns in diplochromosomes created during endoreduplication show that a universal type of epigenetic mechanism must be involved, in which the past order of DNA strands' synthesis is recognized. Any mechanistic explanation of coordinated recognition of one strand across the length of DNA duplex would have implications for our understanding of (1) differentiation of the sisters chromatids during their individualization; (2) coupled to individualization, formation of higher-order structures in mitotic chromosomes; (3) origins of replication definition; (4) cis-regulation in epigenetic processes and (5) asymmetric cell division. Based on the re-analysis of observations related, among others, to endoreduplication process, we propose a mechanism of coordinated strand recognition, in which the formation of asymmetric hemicatenane structures during DNA synthesis is involved. Hemicatenanes have been previously observed in plasmids, viruses and Crenarchaeota, the prokaryotes with the replication mechanism most closely related to eukaryotes. Our microscopic data indicate broad involvement of this and other DNA-based topological restraints in the organization of chromatin architecture.

1322/B480
Chromatin Organization of the Dot Chromosome in D. melanogaster and D. virilis.
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Chromosome 4 of D. melanogaster - also called the “dot” chromosome because of its small size - is predominantly heterochromatic with the exception of its short arm, approximately 1.2Mb that are amplified in polytene chromosomes. Such amplification is generally a feature of euchromatin. However, HP1 and methylation of histone 3 at lysine 9 (H3K9me), marks generally considered to be heterochromatic are associated with the dot chromosome in cytological studies. Thus, the D. melanogaster dot chromosome exhibits an intriguing combination of characteristics associated with either heterochromatin or euchromatin. Study of polytene chromosomes from D. virilis, a species separated by 40-60 million years of evolution, has indicated that the dot chromosome in this species lacks the prominent enrichment with heterochromatic marks observed in D. melanogaster and thus resembles euchromatin based on these biochemical marks. We have carried out chromatin immunoprecipitation (ChIP) studies in several tissues and find that in third instar larvae and fly heads in the genic regions assayed the difference in H3K9me is approximately 2-fold, significantly less than would be expected from the polytene chromosome studies. ChIP-seq experiments confirmed that H3K9me patterns on the D. melanogaster and D. virilis dot chromosomes in these tissues differ by no more than 2-fold, even when non-genic regions are included in the analysis. The results also demonstrate that the difference in H3K9me is specific to the dot chromosomes, and that both dot chromosomes show increased levels of H3K9me compared to autosomes. Experiments are ongoing to determine if the difference in chromatin state observed in polytene chromosome preparations is specific to the salivary glands, and how histone modification patterns differ on these two homologous chromosomes on genes and repeated sequences.

1323/B481
Functional Differences Among Linker Histone Isoforms in Xenopus.
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Developmental isoforms of histone H1 are evolutionarily conserved but poorly understood. Whereas the embryonic isoform H1M is required for proper mitotic chromosome structure in Xenopus egg extracts, somatic isoforms H1A or H10 cannot substitute at the same concentration,
instead causing chromatin compaction during S-phase, and dissociating from chromosomes during M-phase. Nuclear import factors RanBP7 and importin β bind tightly to somatic H1, and their dissociation significantly restores its ability to rescue chromosome architecture. In the embryo, when H1A is normally expressed, it associates with chromosomes throughout the cell cycle. Cdk1 phosphorylation, which is unique to somatic H1, does not alter its function in egg extracts, but may reduce its affinity for mitotic chromosomes in the embryo. Our results reveal distinct regulatory mechanisms among linker histone isoforms, and a specific role for H1M to compact chromosomes during egg meiotic arrest and early embryonic divisions.

1324/B482
**Genetic Analysis of Histone H2A Variant H2A.Z and NCoR/SMRT Co-Repressor Complex in Saccharomyces cerevisiae.**

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In *Saccharomyces cerevisiae*, the histone H2A variant gene *HTZ1* is synthetic lethal with the genes encoding the four core subunits (*SET3, HOS2, SIF2* and *TBL1*) of the Set3 HDAC complex, which is the functional homolog of mammalian NCoR/SMRT co-repressor complex. H2A.Z and the NCoR/SMRT complex play essential roles during stem cell differentiation and metazoan development. Because both are essential for viability, it is difficult to study their genetic interaction in metazoans. To overcome this problem, we mined the genetic interaction between their yeast homologs, where the fundamental biology should be conserved. Through genome-wide suppressor screens, we expanded the *htz1 set3* binary interaction into a network consisting of major chromatin regulators including Hda1 HDAC complex, Swr1 complex, Ubp8-Sgf11 H2B deubiquitination module, proteasome, histone methyltransferase Set1, silent information regulator Sir3, ADA HAT complex and Slx5-Slx8 SUMO-activated ubiquitin E3 ligase complex, most of which have metazoan counterparts. We were able to categorize these suppressor genes into three mechanistic groups based on their distinctive phenotypes when either *SLX5* or *SIR3* is overexpressed. We showed that a subset of the suppressors function at the transcription level and we furthered showed that the suppressors function extensively into the individual interactomes of *HTZ1* and *SET3*. Novel genetic interactions not previously detected through genomic studies based on binary genetic interactions are also revealed by our study. One example is the relationship between the Slx5-Slx8 complex and the ADA HAT complex and we confirmed this genetic interaction biochemically.

1325/B483
**Toxicity of Orthopaedic Nanoparticles.**

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Joint replacement using cobalt chrome (CoCr) metal-on-metal (MOM) implants is increasingly performed in young patients. The hard bearing surfaces generate large quantities of nanometre sized metallic particles (NPs), exposure to which extends over long periods of time. The mechanisms underlying the potential genotoxicity of these particles are not known. To understand the toxicity of NPs, artificial CoCr particles of various sizes, prepared by 2 different methods [pin-on-plate (30 nm) and thermal plasma (20 nm, 35 nm and 80 nm)], were exposed to human BJ fibroblasts at doses from 0.0005 to 500 μm³/cell, for up to 5 days. Commercially available polystyrene latex beads (58 nm) were used as negative control. The viability of cells was assessed 3 and 5 days after exposure by MTT and LDH assays. Potential damage to microtubules, mitochondria, actin and golgi apparatus, and incidence of micronuclei were determined by immunofluorescence following exposure to CoCr particles at 50 μm³/cell for up to 5 days. Genotoxic response was investigated by observing incidence of chromosomal aberrations using multicoulored Fluorescence in situ hybridisation. The viability of cells treated with latex beads was similar to that of untreated cells at all time points. A dose dependent loss of viability was observed in cells treated with 20 and 35 nm CoCr particles, determined by a loss of mitochondrial
function (MTT) after 3 days. Dose dependent damage to the cell membrane (LDH activity) was observed after 3 days in the 30 nm and 80 nm treated cells. Loss of mitochondrial function was observed in all metal particle treated cells after 5 days, accompanied by elevated LDH activity for all particles at 50 and 500μm3/cell. Incidence of micronuclei progressively increased up to 3 days (4-10%) before reducing to 6% at day 5. Cells treated with particles demonstrated depolymerisation of α-tubulin, fragmentation of mitochondria and alterations in actin without damage to the golgi apparatus at all time points. Aneuploidy and ‘clumping’ of chromosomes was observed in metal treated cells compared with untreated cells. These results suggest a complex and diverse dose dependent response indicating cyto- and genotoxicity of the CoCr particles.

1326/B484
Regulation and Function of the REPO-MAN/PP1 Complex in Mitosis.

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Chromosome structure and dynamics are extremely important in mitosis. During mitotic exit, chromosomes undergo a radical de-condensation in an orderly and timely manner in order to give rise to a functional G1 nucleus. In a previous study we have identified the Repo-Man/PP1 complex as a crucial regulator of chromosome architecture during mitosis (Vagnarelli et al. 2006). Repo-Man, a targeting subunit for the Protein Phosphatase 1γ (PP1γ), is nuclear in interphase, diffuse in prometaphase-metaphase and associated with the segregating chromatids from anaphase onset (Trinkle-Mulcahy et al. 2006). Once on the chromosomes, it inactivates an as-yet unidentified substrate, which cooperates with condensin in prometaphase for the compaction of mitotic chromosomes. The complex re-localisation at anaphase onset is regulated by CDK1 and we have identified a cdc2/cyclinB phosphorylation site on Repo-Man that is important for Repo-Man/PP1 complex localisation and activity during mitosis. The Repo-Man non-phosphorylatable mutant at this specific site causes the complex to localise to the chromosomes before anaphase onset and to trigger Histone H3 de-phosphorylation. In order to identify the chromosomal substrates of the Repo-Man PP1 complex, we have conducted pull-down experiments of tagged Repo-Man followed by mass-spectrometry from cells where mitotic exit has been driven by inhibiting CDK1. Our results suggest that Repo-Man is able to interact with chromatin and this interaction is sufficient to initiate deposition of early nuclear envelope components independent of mitotic exit. Vagnarelli P et al.,(2006) Condensin and Repo-Man-PP1 co-operate in the regulation of chromosome architecture during mitosis. Nat Cell Biol. 8:1133-42. Trinkle-Mulcahy L et al, (2006) Repo-Man recruits PP1 gamma to chromatin and is essential for cell viability. J Cell Biol. 172:679-92.

1327/B485
Human NUDT16 is a Novel Nucleotide Pool Sanitizing Enzyme Hydrolyzing (Deoxy)Inosine Diphosphate to (Deoxy)Inosine Monophosphate.

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Nucleotides in living cells play important roles in a variety of biological reactions, however, those often undergo chemical modification such as oxidation and deamination by reactive oxygen/nitrogen species produced in cells. Such modified nucleotides may be toxic for cells if not eliminated from the nucleotide pools, because those may induce mutation, disturb metabolic reactions, or may function as a signal molecule for cellular responses. We have performed screening for modified nucleotide-binding proteins and identified human nucleoside diphosphate linked moiety X (nudix)-type motif 16 (NUDT16) protein as an inosine triphosphate (ITP)/xanthosine triphosphate (XTP)/GTP-binding protein. Purified recombinant NUDT16 has an activity hydrolyzing pyrimidine nucleoside diphosphates to the corresponding nucleoside monophosphates with the highest kcat/Km value for IDP, and to a lesser extent dIDP, among 29 nucleotides examined. Moreover, NUDT16 has a weak (d)ITP hydrolyzing activity towards (d)IMP, a normal precursor for purine nucleotides. siRNA-mediated knockdown of NUDT16...
significantly suppressed proliferation of HeLa MR cells. Confocal immunofluorescence microscopy revealed that NUDT16 is mostly localized in nucleus, and especially in nucleolus in the cells. We thus concluded that NUDT16 is a novel nucleotide pool sanitizing enzyme, which eliminates (d)IDP and (d)ITP from nucleotide pools mainly in nucleus, thus protecting cells from toxic effects of those nucleotides. We are now analyzing cell cycle and levels of inosine nucleotides in DNA, RNA and free nucleotide pools in HeLa MR cells with or without siRNA against NUDT16.

1328/B486
**Heterochromatin is Regulated by Counteracting Effects of the JIL-1 H3S10 Kinase and the Zinc-Finger Protein Su(var)3-7 in Drosophila.**

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Su(var)3-9, a histone methyltransferase, Su(var)2-5, HP1a, and Su(var)3-7, a 1250 residue zinc-finger protein are all inherent components of pericentric heterochromatin and are important factors for silencing of reporter genes by heterochromatic spreading in *Drosophila*. Su(var)3-9 has been shown to catalyze most of the dimethylation of the histone H3K9 residue which in turn can promote HP1a and Su(var)3-7 recruitment. In addition, Su(var)3-9, HP1a, and Su(var)3-7 can directly interact with each other suggesting a model where interdependent interactions between Su(var)3-9, HP1a, Su(var)3-7 lead to heterochromatin assembly at pericentric sites. The essential JIL-1 histone H3S10 kinase is a major regulator of chromatin structure and it functions to maintain euchromatic domains while counteracting heterochromatization and gene silencing. In the absence of the JIL-1 kinase the major heterochromatin markers H3K9me2 and HP1a spread in tandem to ectopic locations on the chromosome arms with the most pronounced increase on the X chromosomes. Here we address the role of the third major heterochromatin component, Su(var)3-7. We show that the lethality but not the chromosome morphology defects associated with the null JIL-1 phenotype to a large degree can be rescued by reducing the dose of the Su(var)3-7 gene and that Su(var)3-7 and JIL-1 loss-of-function mutations have an antagonistic and counterbalancing effect on position-effect variegation. Furthermore, we show that while reducing the dose of Su(var)3-7 also rescues viability of JIL-1 null mutant larvae, H3K9me2 in polytene squashes still spreads to the chromosome arms, strongly indicating that ectopic Su(var)3-9 activity is not a direct cause of lethality. These observations suggest a model where Su(var)3-7 functions as an effector downstream of Su(var)3-9 and H3K9 dimethylation in heterochromatic spreading and gene silencing that is normally counteracted by JIL-1 kinase activity. Supported by NIH grant GM62916.

1329/B487
**Histone H3 Lysine 9 Methylation in the *C. elegans* Germ Line.**

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Histone methylation is prominent feature of chromosomes in the *C. elegans* germ line. As the temporal/spatial gradient of the germ line allows for the simultaneous visualization of proliferating germ cells and germ cells entering and progressing through meiotic prophase, this system provides a means to investigate how distinct covalent chromatin marks relate to each other and to chromosome structure and function. Here, we show that H3K9me2 and H3K9me3 exhibit different localization patterns in both the hermaphrodite and male adult germ lines. While H3K9me2 is enriched on unpaired sex chromosomes and undergoes dynamic changes as germ cells progress through meiotic prophase, we show that H3K9me3 and H3K9me3 modifications are acquired independently. The putative histone
methyltransferase MET-2 is required for the presence of all germ line H3K9me2 but is dispensable for H3K9me3; conversely, the MES-2 HMTase complex is required for much of the germline H3K9me3 but is dispensable for H3K9me2. Whereas germ lines appear largely normal in most met-2 mutant worms, a low but significant fraction of germ lines exhibit meiotic defects in oocytes or expanded/ectopic mitotic zones. These data indicate that MET-2 is essential to ensure the fidelity of chromosome inheritance and a normal spatial organization within the gonad. Our demonstration of the differential localization and independent acquisition of the H3K9me2 and H3K9me3 chromatin marks, together with analysis of met-2; mes-2 double mutants, strongly supports a model in which these two modifications function independently in adult C. elegans germ cells.

1330/B488
BAC TG Express: One Step Method for High-Level, Copy Number Dependent, Position Independent Transgene Expression.
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Transgenes integrated into mammalian chromosomes express at quite variable and typically low levels due to chromosome position effects and repeat mediated silencing. Here we report a simple, one-step procedure for obtaining high level, reproducible transgene expression in mammalian cells. By inserting mini-genes at different locations within a BAC containing a housekeeping gene locus, we obtain copy number dependent, position independent expression with chromosomal insertion of one to several hundred BAC copies, even when the BAC transgenes are inserted into centromeric heterochromatin. This contrasts with the position dependent, copy number independent, and lower expression values observed after directly transfecting cells with the same minigenes, with or without flanking chicken HS4 insulator sequences. We demonstrate that these multi-copy BAC transgenes adapt an open large-scale chromatin structure, as visualized by lac operator / repressor tagging of the BACs, independent of the chromosome integration sites. Two reporter mini-genes embedded within a single BAC show constant expression ratios and higher and more uniform expression levels than plasmid constructs containing both mini-genes, suggesting this method is extendable to simultaneous expression of multiple transgenes. Our hypothesis is that the open chromatin conformation formed by the BAC transgenes shields the embedded minigenes from both chromosome position effects and repeat mediated silencing and is therefore key to the success of this approach. Our method should facilitate creation of cell lines for therapeutic protein production and provide improved single and multi-gene transgenesis.

1331/B489
Promoter Profiling of Immediate Early Genes in Neuroblastoma by Chromatin Immunoprecipitation (ChIP).
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Despite a large body of research, neuroblastoma tragically remains the cause of 6-10% of all childhood cancers and 15% of cancer deaths in children. The variability in survival rates among neuroblastoma patients is correlated with differential expression of neurotrophic receptors between patient tumors. For example neuroblastoma tumor cells expressing high levels of TrkA are receptive to chemotherapeutic treatments. In contrast, tumor cells expressing high levels of TrkB typically have a poor clinical prognosis. One postulated mechanism for the role of TrkB and its ligand, BDNF, in conferring metastatic potential and enhancing therapy resistance is TrkB receptor signaling leading to the expression of immediate early genes and to tumor expansion. Studies in primary cortical neurons revealed that TrkB activation by BDNF generates a signaling cascade that ultimately results in binding of C/EBPα/β heterodimers and NeuroD to the c-Fos promoter. Additionally, C/EBPα and NeuroD form a complex In Vivo that binds the Egr1 and Egr2
promoters through NeuroD. In this study, we treated wild-type neuroblastoma cells and cells overexpressing TrkA or TrkB with neurotrophic factors and chemical inhibitors or siRNA to several key signaling molecules. Chromatin immuno precipitation (ChiP) was then used to investigate the occupancy of C/EBPα, C/EBPβ and NeuroD on several immediate early gene promoters in response to treatment. In response to ligand stimulation, promoter occupancy was altered on several immediate early gene promoters.

1332/B490

Prelamin A Influences Barrier-to-autointegration Factor Nuclear Localization.
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Lamin a is produced as a protein precursor (prelamin A) which is post-transcriptionally modified at its C-terminal region. It has been demonstrated that accumulation of different intermediates of prelamin a processing induces chromatin remodeling. This effect is consistent with chromatin disorganization described in Hutchinson-Gilford progeria syndrome, Mandibuloacral Dysplasia and Restrictive Dermopathy. In order to understand the molecular mechanism involved in prelamin A-induced chromatin remodeling we evaluated the expression and localization of barrier-to-autointegration factor (BAF), a LEM binding protein linking DNA, in prelamin a accumulating cells. In this study we show that the accumulation of different lamin a precursor (non-farnesylated and carboxymethylated farnesylated prelamin A), obtained through prelamin a mutant constructs expression in HEK293 cells, induced BAF relocalization at the nuclear level. The immunofluorescence reaction showed BAF and different prelamin a colocalization at the nuclear periphery as well as at intranuclear aggregates. Western blotting analysis demonstrated that BAF amount did not change in total cellular lysates but it was increased in isolated nuclei of transfected cells. Similar results were obtained in human skin fibroblasts treated with prelamin a interfering drugs. These findings were in accordance with BAF/prelamin a co-immunoprecipitation obtained in HEK-293 coexpressing GFP-tagged BAF and mutated prelamin a constructs. Finally we evaluated the involvement of the inner nuclear membrane protein emerin in prelamin A-dependent BAF nuclear relocalization. In this regard, we treated emerin null human fibroblast from an Emery Dreifuss muscular dystrophy patient with prelamin A-interfering drugs. Immunofluorescence analysis indicated BAF relocalization at nuclear level. In particular, in emerin null cells unprocessable prelamin a was mislocalized showing an honeycomb labeling pattern, and prelamin a aggregates retention at the nuclear periphery. Interestingly, the same mislocalization was shown by BAF.

1333/B491

Human BAHD1 Promotes Heterochromatic Gene Silencing.
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Deregulation of chromatin regulatory factors and/or aberrant chromatin modifications play an important role in a number of human diseases. Recently, several reports pointed to a relationship between chromatin modifications and infectious diseases. During a screen for human proteins that interact with microbial factors and that might play a role in bacterial pathogenicity, we identified a putative chromatin-associated protein, Bromo Adjacent Homology Domain-containing protein 1 (BAHD1). We report here the in-depth characterization of BAHD1 as a novel heterochromatinization factor in vertebrates. BAHD1 interacts with HP1, MBD1, HDAC5 and with several transcription factors. Through electron and immunofluorescence microscopy studies, we show that BAHD1 overexpression directs HP1 to specific nuclear sites and promotes formation of large heterochromatic domains, which lack acetyl histone H4 and are enriched in H3K27me3. Furthermore, ectopically expressed BAHD1 colocalizes with the heterochromatinic X inactive
chromosome (Xi). The BAH domain, which binds the N-terminal tail of histone H3, is required for BAHD1 colocalization with H3K27me3 but not with the Xi. As highlighted by whole genome microarray analysis of BAHD1 knock down cells, BAHD1 represses several proliferation and survival genes and in particular, the insulin-like growth factor II gene (IGF2). We show that when overexpressed, BAHD1 specifically binds the CpG-rich P3 promoter of IGF2, which increases MBD1 and HDAC5 targeting at this locus. This region contains DNA binding sequences for the transcription factor SP1, with which BAHD1 co-immunoprecipitates. Collectively, these findings provide evidence that BAHD1 acts as a silencer by coordinating heterochromatin assembly at specific sites in the genome.

1334/B492
Identification of Genes Involved in Regulation and Modification of Large-Scale Chromatin Structure.
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Increasing evidence points to a possible role of large-scale chromatin structure in regulating gene expression. Identifying proteins which control large-scale chromatin compaction, however, has been difficult. We created a Drosophila S2 cell line in which large-scale chromatin opening of a metallothionein (MT) BAC transgene array, visualized by lac operator/repressor tagging, accompanies MT gene induction. Addition of Cu++ results in an ~2 fold increase in average transgene array size. We are using an RNAi screen to identify candidate genes whose gene products regulate large-scale chromatin structure by measuring BAC transgene array size before and after Cu++ addition. We first tested this approach by RNAi knockdown of metallothionein transcription factor (MTF) and heat shock factor (HSF) genes as positive and negative controls, respectively. MTF knockdown nearly completely eliminated Cu++ induced large-scale chromatin opening while HSF knockdown produced no change in array size relative to untreated cells. We next screened 22 genes coding for subunits of chromatin remodeling complexes followed by an additional 200 genes implicated in chromatin and/or nuclear structure. We observed a high “hit” rate among the chromatin remodeling complex subunits, with knockdown of subunits from multiple chromatin remodeling complexes leading to a more condensed transgene array. From the 200 genes screen we found a number of additional “hits” producing statistically significant decreases or increases in transgene array size versus control cells before and/or after gene induction. Several results point to the general promise of this approach: 1) Multiple examples show similar phenotypes for different subunits from the same complex. 2) Among “hits” showing the most pronounced phenotypes are genes already implicated in mediating possible long-range chromatin effects, including certain polycomb and trithorax related genes, suppressors of PEV, and specific HP1 homologs. 3) Known genetic interactions connect many genes showing strong phenotypes. Secondary screens and additional ongoing work is focused on further validation of our initial results and expanding our RNAi screen.

1335/B493
Actin Filament Nucleation Activity in SW13 Nuclear Extracts.
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The role of actin in the nucleus has been somewhat controversial. is actin simply a subunit of several chromatin remodeling complexes or does it polymerize to form higher order structures that contribute to nuclear function? We have found that crude nuclear extracts from SW13 adenocarcinoma cells nucleate actin polymerization as measured using a standard pyrene actin polymerization assay. The SWI/SNF-like BAF complex may have nuclear actin polymerization activity. The BAF complex is composed of 9-14 subunits, including actin and actin related proteins. The BAF complex has chromatin remodeling activity, shuttles between the nucleus and cytosol, and has been shown to bind actin filaments in a manner possibly analogous to ARP2/3. Immunoaffinity purification, size exclusion chromatography, and inhibitors will be used to...
characterize the actin nucleating activity present in crude nuclear extracts and may indicate whether the BAF complex is involved in nuclear actin polymerization.

1336/B494
Identification and Characterization of Yeast Genes Required for Growth at Low Temperatures.
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A subset of ER-associated degradation genes, UBC7, CUE1 and DOA10, are required for growth of yeast at lower temperatures (<10°C; Loertscher, et. al. 2006, Eukaryotic Cell). To further explore the physiology and genetics of growth at low temperatures, we screened three Saccharomyces cerevisiae deletion mutant collections (haploid, homozygous diploid, and heterozygous diploid) for mutations that resulted in cold sensitivity. Among the 4771 unique genes represented in the combined haploid and homozygous diploid deletion mutant collections, we identified 498 genes that are required for growth at low temperatures. However, 317 of these mutants displayed discrepant phenotypes in the two strain backgrounds. In most cases, the deletion mutation resulted in a cold sensitive phenotype in the haploid but not in the homozygous diploid mutant background. These discrepancies might represent cryptic mutations in the haploid strain that are responsible for the observed cold sensitivity. However, our analysis suggests that at least some of these mutations may result in haploid-specific cold-sensitive phenotypes. We also screened 1121 heterozygous diploid deletion mutants representing essential genes and identified 131 genes that are haploinsufficient for growth at low temperature. In total, we identified 307 genes that are required for growth of S. cerevisiae at 10°C. Analysis of these genes using Gene Ontology bioinformatics resources, demonstrated significant enrichment in the biological processes: vacuolar acidification, threonine biosynthesis, RNA metabolic processes (including rRNA processing and mRNA splicing), and nucleosome organization and histone exchange. We also identified significant enrichment in genes that encode components of protein complexes including the endoplasmic reticulum V-ATPase assembly complex, chromatin remodeling, and actin and tubulin assembly.

Nuclear Import and Export Signals (1337 – 1350)

1337/B495
Small Angle X-ray Scattering of the Yeast Nuclear Export Protein Xpo1p.
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Conformational flexibility is a central feature of the karyopherin proteins that govern the transport of a host of substrates across the nuclear membrane of eukaryotic cells through nuclear pores. These proteins have been shown to be composed of extended arrays of repeated alpha-helical pairs called HEAT repeats. It is via adjustments in the arrangement of these HEAT repeats that karyopherins are able to bind their cargo in one compartment and subsequently release it in the other. The human karyopherin CRM1 (Xpo1p in yeast) mediates export of proteins that contain a leucine-rich nuclear export signal (NES). The small GTPase Ran binds co-operatively to CRM1 together with the NES cargo to generate a three-chain complex. Comparison of the crystal structures of cargo-bound CRM1, with and without RanGTP, to the EM structure of cargo-free CRM1 indicates that CRM1 undergoes a global conformational transition between these states. We have used small angle X-ray scattering (SAXS) to investigate the conformation of the yeast protein Xpo1p bound to various substrates. Ab initio models have been obtained for the wild type protein alone and in complex with binding partners such as the protein Yrb2p and NES peptide. These models show a general shape consistent with the crystal and EM models for the human CRM1 protein.
p110 Retinoblastoma Protein is a Negative Regulator of Nuclear Import of Simian Virus SV40 Large Tumour Antigen.
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Nuclear import of the simian virus SV40 large tumour antigen (T-ag) is dependent on its nuclear localisation signal (NLS) within amino acids 126-132 that is recognised by the importin α/β1 heterodimer, as well as on a protein kinase CK2 site at serine 112 upstream of the NLS, which enhances the interaction c. 50-fold. Here we show for the first time that T-ag nuclear import is negatively regulated by proximal N-terminal sequences (amino acids 102-110) which represent the binding site (BS) for the retinoblastoma (Rb) tumour suppressor protein. Quantitative confocal laser scanning microscopic analysis of the transport properties of T-ag constructs with or without RbBS deletions/point mutations in living transfected cells or in a reconstituted nuclear transport system indicate that the presence of the RbBS significantly reduces nuclear accumulation of T-ag. The analysis of T-ag nuclear import in an isogenic cell pair with/without functional p110Rb and the use of specific antibodies to p110Rb to inhibit T-ag nuclear import In Vitro implicate p110Rb binding as being responsible for the reduced nuclear accumulation, with negative charge at the serine106 phosphorylation site within the RbBS appearing to be responsible for the inhibitory effect. The fact that p110Rb appears to be able to inhibit T-ag nuclear transport has important implications for the regulation of nuclear import of the other proteins from the various other viruses of medical significance that interact with p110Rb, and how this may relate to transformation.

Structural Flexibility of Importin β Facilitates Its Fast and Selective Transport through the Nuclear Pore Complex.
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The nuclear pore complex (NPC) is composed of more than 30 different kinds of polypeptide (nucleoporin, Nup) and mediates macromolecular transport between the cytoplasm and the nucleoplasm. Small proteins (<40 kDa) are able to pass through the NPC by passive diffusion, but large proteins require another set of proteins called transport mediators. Importin β strongly interacts with Phe-Gly (FG) motif in Nups and translocates the cargo protein from the cytoplasm to the nucleoplasm. We demonstrate here that the fast and selective translocation of importin β through the NPC is closely related to the hydrophobic environment of the NPC channel and the amphipathic characteristics of importin β. The surface hydrophobicity of purified karyopherins and nucleoporins was quantified by hydrophobic fluorescent probe (bis-ANS). Almost all of the FG-Nups tested here (Nup54, Nup62, Nup153, Nup358) showed highly hydrophobic characteristics, suggesting that the central channel of the NPC is highly hydrophobic. The hydrophobicity was correlated with the number of phenylalanyl residues. Karyopherins (importin β, CRM1, Cas), as well as other proteins involved in the transport (importin α and β-catenin), also exhibited high hydrophobicity. However, the surface characteristics of importin β changed in different environments; in a cytoplasmic condition (120 mM KCl), importin β showed low hydrophobicity, whereas it became highly hydrophobic in a hydrophobic environment such as in 50% ethanol, suggesting that importin β changes its surface hydrophobicity depending on the environment. The quantitative analysis of the secondary structures of importin β by circular dichroism demonstrated that the surface hydrophobicity change of importin β is caused by the collapse of amphipathic α-helixes (HEAT repeat); in cytoplasmic condition, importin β contains α-helixes at 78%, but in a hydrophobic condition (50% ethanol), the α-helix content dropped to 33%. from all of these results, we propose a new model of protein transport through the NPC.

Oxytocin Induces Nuclear Translocation of its Receptor Followed by Interaction with Specific Transcription Factors in Osteoblast.

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2009 ASCB Regular Abstracts
We demonstrated a direct anabolic effect of oxytocin (OT) on bone. OT upregulates expression of osteoblast markers and transcription factors as Osterix, Schnurri, AFT-4, Osteocalcin, Osteopontin. This effect is mediated by OTR expressed by osteoblast (Tamma 2009). We investigated how OT and OTR promote bone formation and osteoblast differentiation and observed that loss of OTR in primary osteoblast impairs mineralization. A previous work (Kinsey 2007) demonstrated nuclear localization of OTR in neoplastic cells and fibroblasts; accordingly we found the protein in osteoblast nuclear extracts after OT stimulus (15-30 min), suggesting a possible role in regulating transcription and/or transcription factors. By immuneprecipitating, we found physical interaction of native OTR with the osteoblast transcription factor Runx-2 and with the transcription coactivator Schnurri-2, in response to OT stimulus. Furthermore following simultaneous osteoblast stimulation with OT and BMP2, we observed OTR/Smad4 interaction in nuclei. In support of this, chromatine immuneprecipitation indicates a direct binding of OTR to the nuclear chromatin. By confocal immunofluorescence nuclear translocation of the receptors and co-localization with Runx-2 and Schnurri-2 was evident after OT stimulus. Exogenous OTR-EGFP fusion protein transfected in primary osteoblasts, translocates into the nucleus following OT treatment, turning from a diffuse cytoplasmic-membrane pattern to a dots-like nuclear and perinuclear localization. We hypothesize a possible intracrine action of OT in the OTR translocation into the nucleus, since several peptide hormones have been reported to act intracellularly, inducing internalization or nuclear translocation of their receptor (Re RN 2006). We stimulated primary osteoblasts with dLVT-Alexa546, a fluorescent agonist of OT, and observed In Vivo by confocal microscopy up to 2 hrs after treatment how the fluorescent peptide moved from extracellular spaces into the cells, supporting the hypothesis of an OT intracrine action in osteoblasts. OTR nuclear localization and association with transcription factors and DNA could be a possible mechanism for the observed increased osteoblastogenesis induced by oxytocin.

1341/B499
Multiple Mechanisms Control the Nucleolar Trafficking of the Chaperone Hsc70.
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Hsc70 is a constitutively synthesized chaperone which is essential for cell viability and involved in numerous cellular functions. This includes the proper folding of nascent polypeptides, protein targeting to organelles and refolding of damaged proteins. Moreover, hsc70 plays a crucial role in many pathophysiological processes and human diseases such as cancer, damage caused by ischemia/reperfusion, the response to heat stress and other environmental insults. Hsc70 shuttles between the nucleus and the cytoplasm; under non-stress conditions the chaperone is distributed throughout the cell. In response to heat shock, hsc70 accumulates in the nucleoplasm. When cells recover from stress, hsc70 transiently concentrates in nucleoli. Here, we used human culture cells to define at the molecular level the mechanisms that control the intracellular trafficking of hsc70. Several aspects of hsc70 transport were investigated. First, we defined the region of hsc70 that is necessary and sufficient for accumulation of the chaperone in the nucleolus. To achieve this, reporter proteins were generated that contain defined segments of hsc70 fused to the non-nucleolar reporter protein GFP. Confocal microscopy combined with quantitative image analysis was carried out on cells transiently synthesizing GFP-fusion proteins. These studies identified a short segment of the chaperone which is sufficient to mediate stress-dependent accumulation of hsc70 in the nucleolus. Moreover, our research shows that hsc70 carries a composite stress-inducible nucleolar targeting signal that combines constitutive nucleolar targeting with a negative regulatory element. In the absence of stress, this negative regulator prevents hsc70 accumulation in nucleoli. A second set of experiments focuses on the signaling events that regulate hsc70 trafficking to the nucleolus. Our research demonstrates that specific kinase cascades and protein phosphatases control hsc70 accumulation in nucleoli. at present, our studies aim at the identification of hsc70 binding partners in the nucleolus and their
possible role in hsc70 nucleolar accumulation. Supported by CIHR, NSERC, FRSQ, Heart and Stroke Foundation of Canada.

**1342/B500**

One-way Ticket to the nucleus: Phosphorylation within the Nuclear Localization Signal of Human dUTPase Regulates Complex Formation with Importin-Alpha.

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Eukaryotes possess a highly efficient spatio-temporal regulatory mechanism for selective translocation of macromolecules between the cytoplasm and the nucleus. For macromolecules larger than about 30 kDa, the Nuclear Localization Signal (NLS) is responsible for nuclear import. NLS is recognized by karyopherins (importins) that carry cargoes through the nuclear pores. Posttranslational modifications within the NLS may profoundly perturb transport kinetics. The dominant isoform of human dUTPase carries a rather unusual NLS segment. dUTPase is indispensable to preserve genomic integrity, since its lack leads to excessive uracil incorporation into DNA that results in chromosome fragmentation. The dUTPase NLS was reported to be phosphorylated at Ser11 by a nuclear kinase, but the function of this modification was not known. We quantitatively analyzed dynamics of nuclear import of wild type, as well as hyper- and hypophosphorylation mimic point mutant dUTPase species in live HEK cells using videomicroscopy. For the first time, we revealed that phosphorylation of the NLS by a nuclear kinase leads to exclusion of dUTPase from the nucleus. This defines transport as a one-way ticket to the nucleus: A dUTPase molecule can get inside the nucleus only once in its nascent unphosphorylated form. Once inside, it gets phosphorylated. If the cell passes through a mitosis, during which the nuclear envelope is transiently degraded, phosphorylated dUTPase escape to the cytosol and cannot be reincorporated into the nucleus. Phosphorylation-incompetent Ser11Gln point mutants show rapid reaccumulation following mitosis. Complexation of importin-alpha and dUTPase is being investigated by a gallery of biophysical techniques (native gel, SPR, ITC, thermofluor and differential spectroscopies). Native gel evidence shows a strong complex between wild type unphosphorylated dUTPase, but no complex between the hyperphosphorylation-mimicking mutant Ser11Glu and importin. We are crystallizing importin-alpha in complex with wild type and mutant NLS peptides and full-length dUTPase. Other aims are to define the cell-cycle stage wherein phosphorylation occurs and identification of the kinase responsible for the modification.

**1343/B501**

A Functional Map of the Nuclear Pore Complex via High Precision Tracking of Single Molecules.

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All materials entering or exiting the cell nucleus during interphase pass through Nuclear Pore Complexes (NPCs), large transport channels embedded in the nuclear envelope. NPCs allow passive diffusion of small molecules and facilitate transport of various types of larger cargos bound to transport receptors. How NPCs achieve their exquisite selectivity remains unclear. We have developed a single-molecule particle tracking assay, based on custom protein-coupled Quantum Dots (QDs), to study the movement of single large cargos through the NPC. A functional map of the interior of the NPC is created by optically tracking single QDs as they translocate with a mean spatial precision of 8nm and a temporal resolution of 25ms. The single transport trajectories reveal a cargo barrier on the cytoplasmic side of the NPC, providing a first selectivity gate. In the central channel of the NPC, cargos move in a non-directional manner consistent with anomalous subdiffusion inside a finite volume with estimated dimensions of ~40 nm in width and ~55 nm in length. Finally, we show that in the absence of Ran, cargos still
explore the entire volume of the NPC, but have a much lower probability to transit into the nucleus, indicating that Ran acts only on the nuclear side of the NPC to bias import.

1344/B502
**Identification and Characterization of the Multiple NLSs in mRNA Export Factor TAP/NXF1.**
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The physical separation of transcription and translation in eukaryotic cells necessitates the massive transport of RNAs and proteins through the nuclear envelop. TAP (or NXF1), the homolog of yeast Mex67p, is a key mediator of general cellular mRNA export and belongs to a structurally and functionally conserved family of nuclear export factors. Although TAP binds the NPC directly through its C-terminal domains and transport the mRNPs into the cytoplasm in the mRNA export process, the nuclear import of TAP needs NLSs that can be recognized by Kapβs. Previous studies suggested that TAP has a PY-NLS at its very N-terminal disordered region, which acted exclusively through Kapβ2 pathway. However, our quantitative data suggests that TAP-NLS fragments used in previous structural and biochemical studies are only partial low-affinity fragments that are missing energetically significant binding determinants/epitopes. Furthermore, we found that the Kapβ2-specific inhibitor M9M did not mislocalize the endogenous TAP in Hela cells, suggesting that TAP may be imported into the nucleus by multiple Kapβs in addition to Kapβ2. Our In Vitro binding assays showed that TAP has a novel linear NLS for Kapβ1, which is localized in the same disordered N-terminus of TAP as the NLS for Kapβ2. Further mutagenesis studies and quantitative measurements indicated that these NLSs use different binding epitopes. In summary, our studies revealed the multiple pathways involved in TAP import and a more complex regulatory mode of nuclear import.

1345/B503
**Nucleocytoplasmic Transport of the SUMO-Acctivating Enzyme Aos1/Uba2.**
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Posttranslational modification with ubiquitin related proteins of the SUMO (small ubiquitin-related modifier) family regulates fate and function of many proteins by changing interactions with proteins, DNA or other macromolecules. The majority of known SUMO targets are nuclear proteins, however the list of soluble and membrane associated cytoplasmic proteins is expanding. Covalent attachment of SUMO to target proteins requires an enzymatic cascade consisting of an E1-activating enzyme, an E2-conjugating enzyme and one of several E3-ligases. Specific SUMO isopeptidases make this modification reversible and highly dynamic. Consistent with the existence of nuclear and cytoplasmic SUMO substrates, conjugating and deconjugating enzymes are found in both compartments. This raises the question of how these separate enzyme pools are generated. Here we describe the mechanism of nuclear protein import of the SUMO E1 enzyme. In contrast to its relative, the ubiquitin E1 enzyme Uba1, the SUMO E1 enzyme is a heterodimeric complex of the two subunits Aos1 (Sae1) and Uba2 (Sae2). Using site directed mutagenesis we mapped functionally independent nuclear localization signals (NLSs) in both subunits. Pull-down assays, In Vitro nuclear import assays with semipermeabilized HeLa cells, and microinjection of fluorescently labeled recombinant proteins served to show that both subunits interact with - and are imported by - importin alpha Beta. Comparison of the behaviour of the single subunits to preformed E1 complex by gelfiltration analysis and microinjection experiments revealed that import of active Aos1/Uba2 complex is exclusively mediated via the NLS in Uba2. Geiss-Friedlander R, Melchior F. Concepts in sumoylation: A decade on. Nat Rev Mol Cell Biol. 2007 Dec;8(12):947-56. Review.

1346/B504
**Regulation of HIV Replication by the Ubiquitin E3 Ligase Huwe1.**
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The Rev protein of the human immunodeficiency virus-1 (HIV-1) facilitates the nuclear export of intron-containing viral transcripts and it is critical for HIV replication. Rev mediates the switch from early to late patterns of HIV-1 gene expression, and is essential for the formation of infectious virions. In a proteomic screen for proteins that selectively interact with Rev and its target RNA sequence, the Rev-Response-Element (RRE), we identified an E3-ubiquitin ligase, Huwe1. Depletion of Huwe1 by RNAi strongly suppresses the expression of reporter genes mediated by the Rev/RRE pathway, which involves the nuclear export receptor Crm1. In contrast Huwe1 silencing has no effect on genes expressed by the CTE (constitutive transport element) pathway, which is mediated by NXF-1, the receptor that exports most cellular mRNAs. This phenotype is explained by our finding that Huwe1 silencing led to strong loss of Rev protein in reporter cells, even though Rev mRNA was present in the cytoplasm at undiminished levels. Silencing of Huwe1 also inhibited the production of infectious HIV by 10-20 fold. A Rev mutant in which all three lysine residues were mutated to arginines accumulated to control levels in Huwe1 silenced cells, and yielded normal trans-activation. This shows that the loss of Rev in Huwe1 silenced cells is due to highly enhanced ubiquitin-mediated proteosomal degradation of Rev and that Huwe1 negatively regulates an ubiquitin E3 ligase that targets Rev for degradation. We have identified several candidate E3 ligases that are the regulatory targets of Huwe1 in our proteomic analysis, and our functional analysis of these in the context of Rev regulation will be discussed.

1347/B505
Multiple Pathways for Nuclear Import of Murine Nkx.2.2.
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Nkx2.2, like other homeobox proteins, plays essential roles in development. Its homeobox domain contains two positively-charged clusters which function as its nuclear localization signal. but mechanisms of its nuclear import is not clear. We observed that Nkx2.2 carries two independent NLSs which are localized at both N- and C- termini of its homeobox, respectively. Amino acids 128KRKRR132 and 180R184K185R are important for their functions. Meanwhile, Nkx2.2 interacts with impbeta1, imp4, imp7, imp8, imp9 and imp13, respectively and can be imported by these impbeta members in digitoin-permeabilized HeLa cells, suggesting that nuclear import of Nkx2.2 is mediated by multiple pathways.

1348/B506
Nuclear Import of the Baculovirus Autographa Califormica Multiple Nucleopolyhedrovirus.
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Autographa californica multiple nucleopolyhedrovirus (AcMNPV), the archetype of the Baculoviridae family, is an arthropod-specific, enveloped, rod-shaped virus with circular double-stranded DNA genome that replicates in the nucleus of its host cells. Baculoviruses have successfully been used to control pests, and they are also extensively used as a eukaryotic expression system for the production of biologically active proteins. Despite this, many steps of the viral life cycle remain uncharacterized such as the mechanism by which baculovirus delivers its genome into the cell nucleus. Recent studies suggest the role of actin filaments in cytoplasmic trafficking of baculovirus' nucleocapsid towards the nucleus, however the subcellular location at which viral nucleocapsids disassemble further releasing the viral genome into the nucleus is unknown. We have used purified AcMNPV nucleocapsids in combination with electron microscopy to reveal the fate of viral nucleocapsids once inside the cell, and the role of nuclear pore complexes (NPCs) in mediating viral nucleocapsid entry into the nucleus. Purified AcMNPV nucleocapsids were microinjected into the cytoplasm of Xenopus oocytes, a system that contains a high density of NPCs and is widely used to study NPCs and nuclear transport. The fate of the microinjected nucleocapsids was then visualized by electron microscopy after fixation and Epon-embedding of oocytes at different times after microinjection. Using this technique, we have found AcMNPV nucleocapsids docking at the cytoplasmic side of the NPC and crossing the NPC intact.
Conditions that inhibit nuclear transport resulted in an accumulation of intact nucleocapsids at the NPCs. At longer time after microinjection and under permissive transport conditions, intact nucleocapsids were found within the nucleus of the *Xenopus* oocytes. Our data support a model in which the intact AcMNPV nucleocapsid, after being released into the cytoplasm during infection, enters the nucleus through the NPC and disassembles inside the nucleus to release its genome for replication.

1349/B507

**Molecular Recognition of TPX2 by Importin-alpha in Mitosis.**

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During interphase, specific receptors transport cargo molecules between the cytoplasm and nucleus. Recent work has shown that these receptors also have important functions in mitosis where they sequester and release key molecules involved in spindle assembly following nuclear envelope breakdown. This process depends on the receptors specifically recognizing their targets from a large pool of potential binding partners. Although their function has been well characterized in nuclear transport, relatively little is currently known about the molecular details of recognition between these receptors and key molecules that mediate mitotic spindle formation. Cargo proteins designated for import have characteristic nuclear localization sequences (NLSs), which contain either one (monopartite) or two (bipartite) patches of basic residues. These residues are recognized by the adaptor, importin-alpha. Two NLS-binding sites have been identified on the surface of importin-alpha. The major pocket binds classical monopartite NLSs, whereas bipartite NLSs bind to both sites. We have characterized the molecular recognition of the NLS of the spindle assembly factor TPX2, a microtubule-associated protein that is sequestered by importin-alpha. A combination of biochemical, biophysical and structural methods was used to analyze the binding of TPX2 to importin-alpha. A 2.1 Å resolution crystal structure of TPX2 bound to importin-alpha indicated that the TPX2 NLS binds to Arm repeats 7-9 of importin-alpha, which represents the minor NLS-binding site. Pull-down assays with importin-alpha variants that interfere selectively with the binding to each site confirmed that the TPX2 NLS binds to the minor pocket of importin-alpha. To characterize the binding of the TPX2 NLS to Arm repeats 7-9 of importin-alpha, we determined the binding affinity of this interaction and show that the binding occurs with high affinity. Taken together, these data represent the first example of a naturally-occurring NLS that binds primarily to the minor NLS-binding site of importin-alpha and provide insights into the basis of selectivity for recognition of the TPX2 NLS during mitosis. Supported by a Wellcome Trust Programme Grant to MS.

1350/B508

**The Oligomerization and Nucleocytoplasmic Transport of NTF2 Probed by Two-photon Activation and Fluorescence Fluctuation Spectroscopy.**

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Large proteins and macromolecular complexes have to enter and leave the nucleus in an efficient and selective manner. Macromolecules that are greater than 40 kDa are transported actively across the nuclear envelope through nuclear pore complexes using soluble transport factors or carrier molecules that cycle between the cytoplasm and nucleus. The carrier proteins themselves interact with each other in order to transport cargo proteins across the nuclear pore complexes. In this work, we apply dual-color time-integrated fluorescence cumulant analysis (TIFCA), a fluorescence fluctuation spectroscopy technique, to investigate the protein interactions of the carrier proteins directly in cells. In addition, we apply two-photon activation to directly examine the nucleocytoplasmic transport of photoactivatable GFP tagged carrier proteins. With these two approaches, we are able to probe the nucleocytoplasmic transport process of NTF2 directly inside cells and under equilibrium conditions. Our data indicate that NTF2 forms a much tighter dimer inside living cells than has been reported from In Vitro data. However, the measured transport properties across the nuclear pore complex are not consistent with dimeric NTF2. We will discuss the implications of these results for models of nucleocytoplasmic transport.
**Signal Transduction II (1351 – 1375)**

**1351/B509**  
*Where the Signal Begins - Studies on Local Dynamics of Calcium Transients in Glioma C6 Cells.*  
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Here in a single-cell calcium visualization system we use for the first time a fast protocol to see subcellular dynamics of calcium response in glioma cells. The signal is evoked by UTP via stimulation of G coupled metabotropic P2Y2 nucleotide receptors, and activation of PLC. Calcium release from endoplasmic reticulum in the IP3-dependent manner. Glioma C6 is a well-known model of non-excitatory cells coming from central nervous system. We use fura 2-AM very popular ratiometric dye. The use of ratiometric technique ensures us that calcium-dependent changes in the very flat parts of the cell will not disturb the results, what may happen during confocal calcium measurements when a slice is thicker than lamellipodium. Sub-second measurement is sufficient to observe that calcium response begins in one region situated on the edge of the cell and is spread to the whole cell body within couple of seconds. However depending on the position of measurement point: in lamellipodium area or in the cell center, we can see different dynamics of the local response. for every time series two parameters are estimated: signal delay (from the first sign of intracellular signal initiation to the signal initiation in the given cell point) and local signal growth rate. In the cell center we observe longer delay in comparison to the signal initiation region. Additionally, in about third of the cells, the signal growth is the fastest in the initiation region. We have found that initial, IP3 dependent, signal generation is usually located in cell flat lamellipodium and then signals spread into the whole cell body. The second phase of signal formation, entrance of calcium into the cell by store operated calcium channels is evenly distributed through the cell. The finding of the local character of the calcium signal generation show the new light on the phenomena like nucleotide dependent cell chemotaxis observed widely in the glial cells.

**1352/B510**  
*The Tyrosine Phosphatase, SHP-1, Acts on Multiple Tyrosine Kinase Receptors to Negatively Regulate Human Cytotrophoblast Proliferation.*  
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Pregnancy complications such as fetal growth restriction are associated with abnormal placental cell (cytotrophoblast; CT) proliferation and apoptosis. Regulation of these events is unclear but recently we have used a placental explant model to demonstrate that IGFs influence CT kinetics. IGF activation of the IGF1R is modulated by protein tyrosine phosphatases (PTPs); using siRNA we have shown that the PTP, SHP-2, is required for IGF function but analysis of PTP activity following SHP-2 knockdown revealed that SHP-2 accounts for only 20% of total PTP activity in placenta, thus other PTPs must be important. mRNA for a closely related PTP, SHP-1, is present in placenta but its actions are unknown; in other systems it negatively regulates signalling events. This study explored the hypothesis that SHP-1 regulates trophoblast proliferation. SHP-1 or non-targeting siRNA (500nM) was delivered to BeWo choriocarcinoma cells or first trimester villous tissue fragments and both were maintained in culture for 72 h then treated with IGF-I or -II (10nM) for a further 24 h. Western blot, immunohistochemical (IHC) and QPCR analysis revealed a significant reduction in SHP-1 expression (85% in BeWo cells; 73% in explants). IHC analysis of cell proliferation (Ki67) demonstrated that SHP-1 knockdown had no effect on IGF-induced proliferation but basal (serum-free) proliferation in both BeWo cells and explants was enhanced (from 19.7.4±2.6% to 52.3±2.9% and 22.3±3.7% to 63.8±2.7% respectively; both P<0.05, n=4). The potential mechanism(s) by which SHP-1 regulates basal proliferation were elucidated using antibody arrays and IHC to examine the activation status of multiple receptor tyrosine kinases (RTKs) in SHP-1-depleted cells; following SHP-1 knockdown the activation of several RTKs, notably EGFR and TrkB, was enhanced suggesting that under basal conditions, SHP-1 may
interact with these molecules to inhibit cytotrophoblast proliferation. This study demonstrates a role for SHP-1 in human trophoblast and establishes SHP-1 as negative regulator of multiple RTKs that regulate placental growth. Modulation of SHP-1 expression/activity may provide a strategy for correcting altered trophoblast proliferation in pregnancy complications.

1353/B511
**Normoxic Upregulation of Hypoxia-Inducible Factor-1α by the EP1 Prostanoid Receptor Involving Activation of a PI3K-Akt-mTOR Signaling Pathway.**

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The EP1 prostanoid receptor is one of four subtypes whose endogenous physiological activator is prostaglandin E2 (PGE2). EP1 receptors are in the family of G-protein coupled receptors and are known to activate Ca\(^{2+}\) signaling. In HEK cells stably expressing human EP1 receptors (HEK-hEP1) we have found that PGE2 can upregulate the expression of hypoxia inducible factor-1α (HIF-1α) under normoxic conditions. HIF-1α is an important transcription factor that is classically upregulated during hypoxia by a mechanism involving decreased protein degradation. The purpose of this study was to examine the mechanism of the EP1 receptor mediated upregulation of HIF-1α. Immunoblot analysis was used to examine the expression of HIF-1α in HEK-hEP1 cells following pretreatment with various signaling pathway inhibitors and stimulation with 1 μM PGE2 for 6 hrs. Pretreatment of cells with pertussis toxin (G\(_{\text{i/o}}\) inhibitor), wortmannin (phosphoinositide-3 kinase inhibitor), Akt inhibitor, or rapamycin (mTOR inhibitor) all inhibited the PGE2 stimulated upregulation of HIF-1α. Immunoblot analysis also showed that PGE2 could stimulate the phosphorylation of ribosomal protein S6 (rpS6) in HEK-hEP1 cells, reflecting mTOR-mediated activation of S6 kinase. This phosphorylation of rpS6 could be inhibited by pretreatment with pertussis toxin or wortmannin. Quantitative real time PCR showed that mRNA levels of HIF-1α were unchanged following stimulation of HEK-hEP1 cells with PGE2, indicating that the upregulation of HIF-1α does not involve increased transcription. Stimulation of endogenous EP1 receptors in human hepatocellular carcinoma cells (HepG2) essentially recapitulated the findings obtained with HEK-hEP1 cells. We conclude that human EP1 prostanoid receptors can upregulate the expression of HIF-1α under normoxic conditions through increased translation by coupling to G\(_{\text{i/o}}\) and activation of a PI3K-Akt-mTOR signaling pathway. These findings have significance for understanding the role of PGE2 and the EP1 receptor in tumor growth and metastasis.

1354/B512
**TGF-β-induced Shc Signaling Persists in the Absence of the Type II Receptor.**

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TGF-β signaling is initiated by the sequential activation of Type II and Type I receptors. The Type I receptor then serine phosphorylates and activates Smad proteins. However, both TGF-β receptors also possess a tyrosine kinase activity that enables them to directly bind and phosphorylate Shc adapter proteins, thereby initiating the well-characterized Shc signaling pathway leading to Erk MAP kinase activation. Although Shc and Smad proteins are both phosphorylated by the TGF-β receptor complex, deletion of individual TGF-β receptors may not disrupt their activation to the same extent. We hypothesized that TGF-β-induced Shc signaling would persist in cells that are deficient in TGF-β Type II receptors (TβRII). Palatal fibroblasts were isolated from E13.5 fetuses produced by mating TβRII flx/flx females with TβRII flx/++; wnt1-Cre male mice. TβRII is absent from the palatal mesenchyme in 25% of the conceptual products of this mating. Fetuses were genotyped by PCR. Cells were passaged for one week, starved overnight, and then stimulated with TGF-β1 4 ng/ml for up to 20 minutes. Smad signaling, as assessed by Smad2 phosphorylation, was abrogated. However, the activation of Shc, Erk1, and Erk2 persisted. Erk phosphorylation was decreased in the same cells when transfected with
siRNA against Shc, suggesting that Erk activation was dependent on Shc. These results suggest that the two signaling pathways initiated by TGF-β have different requirements for the Type II receptor.

1355/B513
Synthesis of Novel Fluorescent GTP Analogue and Its Interaction with Small G-protein Ras.
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A new fluorescent GTP analogue, 2'(3')-O- {6-(N- (7-nitrobenz-2-oxa-1, 3-diazole-4-yl) amino) hexanoic}-GTP (NBD-GTP) was synthesized in order to apply to the kinetic study for Ras (GTP binding protein). NBD-GTP was synthesized by coupling of GTP and 6-(N- (7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-amino) hexanoic acid (NBD acid) according to the similar method of NBD-ATP which we have previously reported. The products were purified by HPLC on an RP-C18 column. The fluorescence spectrum of NBD-GTP in buffer (pH7.5) showed an excitation maximum at 471 nm and an emission maximum at 534 nm. The fluorescence intensity of NBD-GTP increased slowly by 120% when Ras was added to the NBD-GTP solution. In the co-presence of regular GTP, the enhancement of NBD-GTP fluorescence significantly reduced, suggesting that NBD-GTP binds to the GTPase site. We also prepared the GTP free Ras by treating with EDTA. Initial burst of fluorescence enhancement of NBD-GTP and subsequent slow increase were observed on the interaction of NBD-GTP and GTP free Ras. The results may suggest that the initial burst of fluorescence reflects the binding of GTP to the free GTPase site of Ras, and the slow phase corresponds to the formation of Ras-GDP-Pi state. The novel fluorescent GTP analogue NBD-GTP may be applicable to the kinetic study for G-protein. 1) Maruta S, Mizukura Y, Chaen S. (2002) J Biochem. 131, 905-11.

1356/B514
The Yeast Endocytic Protein Epsin-2 Functions in a Cell Division and Septin Organization Pathway.
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The epsins are a family of adaptors involved in recruiting other endocytic proteins, binding of ubiquitylated cargo and induction of membrane curvature. These molecules bear a characteristic Epsin N-Terminal Homology (ENTH) domain and multiple peptide motifs that mediate protein-protein interactions. We have previously demonstrated that the ENTH domain of epsin is involved in Cdc42 signaling regulation. Here, we present evidence that yeast epsin-2 (Ent2) plays a signaling role during cell division. We observed that overexpression of the ENTH domain of Ent2 (ENTH2), but not Ent1, promoted the formation of chains of cells and aberrant septa. This dominant-negative effect resulted from ENTH2-mediated interference with septin assembly pathways. We mapped the ENTH2 determinants responsible for induction of the phenotype and found them to be important for efficient binding to the septin regulatory protein, Bem3. Supporting a physiological role for epsin-2 in cell division, the protein localized to sites of polarized growth and cytokinesis and rescued a cell division defect induced by Bem3 misregulation. Collectively, our findings provide a potential molecular mechanism linking endocytosis (via epsin-2) with signaling pathways regulating cell division.

1357/B515
A Complex of Plectin and Dystroglycan Mediates Mechano-Signal Transduction in Lung Epithelial Cells.
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In the normal airway, mechanical forces regulate fetal lung growth and maturation and impact airway epithelial cell growth and differentiation in the adult via effects on signaling pathways. However, inappropriate mechanical stimulation of the lung may result in increased membrane permeability, pulmonary edema and inflammation. Our research goal is to dissect how mechanical stimuli at the surface of alveolar lung cells (AECs) modulate signal pathways. Previous work has established that the cell surface proteoglycan dystroglycan (DG) is required for cyclic stretch-induced activation of two signaling cascades: one involving the extracellular signal-regulated kinases 1 and 2 (ERK1/2) and the other involving the adenosine 5'-monophosphate-activated protein kinase (AMPK). These pathways appear to be protective and inhibit the generation of reactive oxygen species following mechanical stimulation. In this study we tested the hypothesis that plectin, a large cytoskeletal cross linker that also acts as a scaffolding protein, may be downstream of DG and mediates the conversion of mechanical (stretch) signals acting on the outside of AECs into chemical signals in the cytoplasm of these cells. Immunoprecipitation assays of extracts of AECs revealed that plectin and DG assemble as a complex. We next knocked down plectin expression in AECs by up to 85% using adenoviral shRNA and found no obvious impact on the cytoskeleton or adhesion. When the same cells are subjected to 10 minutes of cyclic stretch (30 cycles per minute with stretching-relaxation ratio of 1:1, resulting in 10% linear elongation of the membrane as measured microscopically), we found that the activity levels of ERK1/2 and AMPK were reduced by up to 50% compared to cells treated with control shRNA. Together, these data indicate that a functional complex of DG and plectin is necessary for the transmission of mechanical signals in AECs and likely plays a role in ameliorating the damage when inappropriate forces are exerted on the lung during mechanical ventilation.

1358/B516

Investigating the Mechanism(s) Underlying Selective Resistance of Skin Cells Versus Immune Cells in IDO-Induced Low Tryptophan Environment.

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Introduction: Indoleamine 2, 3-dioxygenase (IDO), a tryptophan degrading enzyme, is a potent immunomodulatory factor. We have previously shown that IDO expression in fibroblasts selectively induces apoptosis in bystander immune cells but not in primary skin cells such as fibroblasts and keratinocytes. However, the mechanism(s) by which skin cells, but not immune cells, are resistant to IDO-induced low tryptophan environment is not elucidated. Objective: to study whether the activity of general control non-derepressible-2(GCN2) kinase stress-responsive pathway and its recently known inhibitor, protein IMPACT homolog, in skin and immune cells is regulated differentially in response to IDO-induced low tryptophan environment. Methods: IDO-expressing human fibroblasts were co-cultured with bystander Jurkat cells, human T cells, fibroblasts or keratinocytes for 72 hours. The levels of phosphorylated GCN2, total GCN2 and IMPACT were evaluated by Western blot analysis. Methyl thiazolyl tetrazolium (MTT) and viability assay were performed for IMPACT small interfering RNA-knocked down fibroblasts co-cultured with IDO-expressing fibroblasts. Appropriate control groups were allocated. Results: Activation of GCN2 kinase pathway was significantly higher in immune cells, when exposed to IDO, in compared with that of skin cells. on the other hand, the IMPACT protein was highly and constitutively expressed in primary skin cells at both message and protein levels while its expression level was very low in T cells and undetectable in Jurkat cells. IMPACT expression level was independent of IDO-induced environment. on the other hand, IMPACT expression was upregulated in a time dependent manner in fibroblasts cultured in tryptophan-free media. A significant IDO-induced suppressive as well as apoptotic effect was demonstrated in IMPACT
knocked down fibroblasts co-cultured with IDO-expressing fibroblasts. Conclusions: Our findings suggest for the first time that high expression of the protein IMPACT homolog in non-immune cells such as skin cells acts as a protective mechanism against IDO-induced GCN2 activation therefore makes skin cells resistant to the amino acid-deprived environment caused by IDO.

1359/B517
Autocrine Motility Factor/Phosphoglucose Isomerase (AMF/PGI) Regulation of the ER Stress Response and Cell Death.
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Phosphoglucose isomerase (PGI) is an ubiquitous multifunctional protein that plays important roles in metastasis, and is equivalent to the cancer associated cytokine, autocrine motility factor (AMF). Secreted AMF promotes cancer cell metastasis by stimulating cell motility in an autocrine manner after binding to its 78 kDa seven-transmembrane glycoprotein receptor/autocrine motility factor receptor (gp78/AMFR). Gp78/AMFR is a cell surface receptor that is also localized to a mitochondria-associated endoplasmic reticulum (ER) subdomain where it acts as an ER membrane-anchored ubiquitin ligase (E3) involved in ubiquitination of ER proteins and ER-associated degradation (ERAD). AMF/PGI prevents the ER stress response (cytosolic Ca2+ concentration, the expression of Bip, CHOP and p-ERK) and cell death (mitochondrial cytochrome C release, expression of cleaved caspase-3 and PI/annexin V labeling) induced by thapsigargin-induced disruption of Ca2+ homeostasis or Ca2+ independent tunicamycin-induced inhibition of protein glycosylation. AMF/PGI has previously been shown to promote cell survival partially via a PI3K/Akt pathway. AMF/PGI increased pAkt activation but the PI3K inhibitor LY294005 only partially reversed the effect of AMF/PGI on the ER stress response. AMF/PGI treatment does not affect Chop expression in gp78/AMFR knockdown Hek293 cells compared with non-targeted control cells. Furthermore, Chop expression in Flag-tagged gp78/AMFR transfected cells is reduced relative to cells transfected with a gp78/AMFR mutant deficient in ubiquitin ligase activity. AMF/PGI interaction with its receptor gp78/AMFR therefore performs a crucial function in protecting cells against ER stress via activation of pAkt activity but also via other mechanisms that may involve gp78/AMFR ubiquitin ligase activity.

1360/B518
Glycogen Synthase Kinase-3 and Omi/HtrA2 Induce Annexin A2 Cleavage Followed by Cell Cycle Inhibition and Apoptosis.
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Annexin A2 is involved in multiple cellular processes, including cell survival, growth, division, and differentiation. Lack of annexin A2 makes cells more sensitive to apoptotic stimuli. Here we demonstrate a potential mechanism for apoptotic stimuli-induced annexin A2 cleavage, which contributes to cell cycle inhibition and apoptosis. Annexin A2 was persistently expressed around the proliferative but not the necrotic region in BALB/c nude mouse with human lung epithelial carcinoma cell A549-derived tumor. Knockdown expression of annexin A2 caused cells susceptible to either serum withdrawal-induced cell cycle inhibition or cisplatin-induced apoptosis. Under apoptotic stimuli, annexin A2 was cleaved via a time-dependent manner. Under apoptotic stimuli, glycogen synthase kinase-3 (GSK-3) was activated following Akt inactivation. Treating cells with GSK-3 pharmacological inhibitor SB415286, small interfering RNA (siRNA), and dominant mutant, showed a blockade on annexin A2 cleavage. Inhibiting okadaic acid-sensitive protein phosphatase 2A (PP2A) blocked Akt inactivation, GSK-3 activation, and annexin A2 cleavage. Under normal growth condition, inhibiting phosphatidylinositol 3-kinase/Akt signaling
only using LY294002 could not induce annexin A2 cleavage while GSK-3 was activated, suggesting the potent role of PP2A. Furthermore, inhibiting serine proteases blocked apoptotic stimuli-induced annexin A2 cleavage. Blockage of PP2A and GSK-3 reduced Bax translocation to mitochondria and Mcl-1 degradation. Bax knockdown expression or Mcl-1 overexpression prevented annexin A2 cleavage. Mitochondrial serine protease Omi/HtrA2 was activated and relocated to the cytosol, while GSK-3 inhibition blocked these phenomena. Notably, Omi/HtrA2 inhibitor and siRNA blocked annexin A2 cleavage. Therefore, inhibiting GSK-3 reversed serum withdrawal-induced cell cycle inhibition or cisplatin-induced apoptosis. Taken together, these results demonstrate that GSK-3 is interrelated with Omi/HtrA2 in causing annexin A2 cleavage followed by cell cycle inhibition or apoptosis.

1361/B519
Investigation of the Dual Tyrosine Phosphatase-3 Roles in DNA Damage Response and Cell Cycle.
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Objectives: This project investigates new functions and targets to the atypical dual tyrosine phosphatase 3 (DUSP3 or VHR) in ionizing radiation induced foci (IRIF) of HeLa cells, exposed to gamma or UVC ray and proliferating at different phases of cell cycle, aiming to identify possible involvements of this enzyme in DNA repair and genomic instability. Methods: Experimental approaches were based in: 1) Western Blots to VHR and Actin proteins for checking expression and stability along cell cycle; 2) Fluorescence Confocal Microscopy to VHR, pJNK, pH2AX, and other proteins (from next item) for determination of expression and (co-)localization, and 3) theoretical investigation of structural features of proteins known to be involved in DNA damage response and repair for further comparison with known VHR substrates by using bioinformatic tools. Results and Conclusions: VHR is highly expressed protein and very stable to degradation that quickly migrates to nucleus of HeLa cells after low (0.5Gy) to high (20Gy) doses of gamma radiation, which does not affect VHR expression but strongly reduces cell survival. on the IRIFs, VHR co-localizes with pH2AX and, after UVC radiation of these cells, with p-JNK and also pATF2, but exclusively at interphase. Alignment analysis raised a list of more than 30 putative substrates for DUSP3 involved in DNA damage and repair, and one of those, the Mre11 protein, was confirmed to co-localize with VHR in IRIF by Confocal Microscopy. In addition, different tumorigenic human cell lines and VHR substrates under investigation have revealed a novel and unknown role for VHR in genotoxic stress, besides dephosphorylation of ERK and JNK. (Financial Support: FAPESP, CNPq).

1362/B520
Cpc2 Plays a Role in Cross-tolerance to Environmental Stress.
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Eukaryotic cells have evolutionarily elaborated stress responses to adaptively deal with the ever-changing environment for their survival and efficient growth. Treatment of cells with low level of stress (sublethal stress) leads to transient resistance to higher level of the same and other forms of stress. These phenomena are known as acquired stress tolerance or cross-tolerance, respectively. They are observed in a wide variety of organisms from yeast to human, suggesting its phylogenetic conservation. However, their molecular mechanisms are still not fully understood. To identify factors that function in such adaptive responses, we set out a genetic screen for fission yeast mutants defective in cross-tolerance. We have isolated five candidates and identified cpc2 as the responsible gene for three of them. Cpc2 is a highly conserved protein (homolog of mammalian RACK1), which is predicted to function as a scaffold to regulate signal transduction and to be involved in translational control. Translation initiation factor 2 α-subunit (eIF2α) is phosphorylated in response to cellular stress, which causes a reduction in the global translation
rate and an increase in the selective translation of proteins that are required for cell survival under stress. We found that eIF2α phosphorylation upon stress treatment was decreased in Δcpc2 cells. Furthermore, cpc2 and gcn2, that encodes eIF2α kinase, exhibited genetic interaction. Stress-activated MAP kinase (SAPK) Sty1/Spc1 pathway is crucial for induction of stress-responsive genes through the transcription factor Atf1. We found that the kinetics of Atf1 phosphorylation was delayed in Δcpc2 cells. Consistently, the mRNA expression of some stress-responsive genes was decreased. These data suggest that Cpc2 is a key molecule in adapting to environmental changes by regulating both SAPK-dependent transcriptional response and eIF2α kinase-dependent translational response.

1363/B521
**The Pseudophosphatase MK-STYX Interacts with G3BP and Decreases Stress Granule Formation.**

MK-STYX is a pseudophosphatase member of the dual specificity phosphatase sub-family of the protein tyrosine phosphatases. MK-STYX is catalytically inactive due to the absence of two amino acids from the signature motif that are essential for phosphatase activity. The nucleophilic Cys residue and the adjacent His, which are conserved in all active dual specificity phosphatases, are replaced by Ser and Phe, respectively, in MK-STYX. Mutations to "restore" the active site His and Cys residues generated a form of MK-STYX that displayed robust enzymatic activity. Using mass spectrometry, we identified Ras-GTPase activating protein SH3 domain binding protein-1 (G3BP1), a regulator of RAS signaling, as a binding partner of MK-STYX. We observed that G3BP1 bound to native MK-STYX; however, binding to the mutant, catalytically active form of MK-STYX was dramatically reduced. In addition to its interaction with RAS-GAP, G3BP1 is an RNA-binding protein with endoribonuclease activity. G3BP1 is recruited to "stress granules" (SGs) after stress stimuli and can induce formation of SGs itself following overexpression. SGs are large structures in which untranslated mRNAs accumulate and may serve as sites of mRNA sorting. We have shown that expression of MK-STYX inhibits G3BP-induced stress granule formation; however, expression of the catalytically active mutant MK-STYX led to an accumulation of smaller, intermediate-sized aggregates, suggesting that the active mutant is impaired in its ability to inhibit stress granule assembly. These data reveal a novel facet of the function of a member of the PTP family, illustrating a role for MK-STYX in regulating the ability of G3BP1 to integrate changes in growth factor stimulation and environmental stress with regulation of protein synthesis.

1364/B522
**Development of an Assay to Quantitate Beta-Catenin Expression and Distribution via Automated Cellular Imaging and Analysis.**
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Canonical Wnt signaling via β-catenin regulates expression of several genes and is perturbed in many cancers and other diseases. In the absence of a Wnt ligand, β-catenin is assembled at cell junctions or phosphorylated by GSK-3 and CK1, leading to its degradation. Upon Wnt binding, phosphorylation and degradation of β-catenin cease, leading to accumulation and nuclear translocation. β-catenin inhibitors are promising drug candidates, notably for cancer. Our objective was to develop an image-based assay for quantitation of β-catenin. A panel of β-catenin antibodies was screened in HeLa, HepG2 and A549 cells. Use of image analysis algorithms to segment nuclear/cytoplasmic regions and cell borders enabled reproducible characterization of signal localization/intensity, antigen specificity and expression changes, allowing selection of an optimal antibody for use in further assay development. Treatment of HeLa cells with serial dilutions of GSK-3 inhibitors IX and X (GSK IX/X) for 24h (10μM max) displayed dose-dependent activation of nuclear and cytoplasmic β-catenin. Increases in nuclear intensity vs. DMSO control...
were robust, showing activations of >30% for 10μM GSK X. HeLa cells undergoing 24h 10μM GSK X treatment were used to evaluate the effects of serum (0, 2, 10%) on nuclear β-catenin activation. A549, HeLa and HepG2 cells were further employed to generate cell type-specific dose response curves for GSK IX/X, as well as for a potent, selective Wnt agonist. By calculating signal:background ratios and nuclear activation for treated cells, we demonstrated that the assay reagents exhibit benchtop stability of >24h. This property greatly facilitates large-scale screening. In summary, we describe the development of a robust assay for β-catenin screening via quantitative imaging. The assay utilizes sensitive, specific immunofluorescent detection of β-catenin, and may be used to rapidly visualize and quantify expression of this protein in a variety of cell types. We demonstrate applicability of this technique to β-catenin localization and site-specific quantitation, generation of dose response curves for modulators of the Wnt/β-catenin signaling pathway and amenability to large-scale screening operations.

1365/B523
Novel Roles of Branched-Chain Fatty Acids in Regulating Post-Embryonic Growth and Development in C. elegans.

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Growth and development of animals are regulated by signaling systems that sense the availability of nutrients and metabolic status. We have shown that a monomethyl branched-chain fatty acid (mmBCFA), C17ISO, a product of leucine catabolism, is essential for postembryonic development. Worms depleted of C17ISO arrest uniformly in L1 prior to the 1st M cell division. We show that C17ISO acts in a novel mechanism in parallel to DAF-2/DAF-16, and that the two pathways converge on the expression of cki-1 that represses cell cycle. We also show that C17ISO homeostasis is regulated by an SREBP-1c-mediated specific feedback mechanism. C17ISO may act as a nutritional factor in a regulatory mechanism of post-embryonic growth and development. To identify factors of the C17ISO-involved pathway, we carried out screens for suppressors of L1 arrest caused by C17ISO depletion. We obtained 9 suppressors of 3 classes: (A) 2 suppressors recovered the ability of the elo-5(-) strain to synthesize C17ISO, likely due to changes in other enzymes to compensate for the loss of ELO-5; (B) 3 suppressors, all alleles of one gene, permitted the C17ISO-depleted animals to pass L1 arrest, but not further growth in subsequent generations without mmBCFA supplement; and (C) 4 suppressors permitted C17ISO-depleted animals to continuously grow without mmBCFA supplement. We showed that the gene (TAT-2) defined by class (B) suppressors encodes one of the P-type ATPases/flippases implicated in mediating phospholipid bilayer asymmetry. We showed that TAT-2, but not other flippases, has a specific role in antagonizing the activity of mmBCFAs in intestinal cells. We found that mutations in tat-2 also suppress the lethality caused by inhibiting sphingolipid biosynthesis. We further showed that the fatty acid side-chains of glycosylceramides contain 20-30% mmBCFAs and that this fraction is greatly diminished in the absence of mmBCFA biosynthesis. Genetic mapping indicated that class (C) mutations are alleles of at least 3 different genes that may potentially define negative factors acting downstream of C17ISO to regulate L1 growth. Molecular analysis of these genes should provide insight to the mechanism of this important mmBCFA functions.

1366/B524
Proteomic Analysis Reveals Novel Binding Partners of Active RhoA.

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RhoA small GTPase is the molecular switch for certain extracellular signals, and implicated in various cellular functions, including cell contraction, cell migration, cell adhesion, vesicular transport, lipid signaling, cell cycle progression, and gene expression. RhoA regulates these
functions through its specific effectors such as Rho-kinase/ROCK and mDia. Abnormal activation of RhoA has been observed in major cardiovascular diseases and cancer. However, the molecular functions of RhoA are not fully elucidated. To address this question, we comprehensively isolated RhoA-interacting molecules from rat heart lysate by affinity column chromatography and determined their molecular identities by MALDI-TOF MS and LC-MS/MS. We identified many proteins that specifically interacted with the GTP-bound active form of RhoA but not with the GDP-bound inactive form. Some of them appear to be novel effectors of RhoA, which include acetyl-Coenzyme a acetyltransferase 1 (ACAT1), inositol polyphosphate phosphatase-like 1 (SHIP2), filamin a interacting protein 1 (FILIP1), and striatin (calmodulin binding protein 3). These results suggest that our proteomic approach is powerful for comprehensively screening novel RhoA-effectors.

1367/B525
MST2- and Furry-mediated Activation of NDR1 Kinase is Critical for Precise Alignment of Mitotic Chromosomes.
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The precise alignment of chromosomes on the metaphase plate before onset of anaphase is essential for ensuring equal segregation of sister chromatids into two daughter cells, and its imperfection potentially causes genomic instability and tumor progression. Nuclear Dbf2-related kinase 1 (NDR1) is an evolutionarily conserved protein kinase, whose activity is regulated by mammalian sterile20-like protein kinase (MST), Furry (Fry) and MOB proteins. Although the NDR1 signaling pathway is implicated in cell division and morphogenesis in yeast and invertebrates, little is known about its functional roles in mammalian cells. In this study, we showed that NDR1 is required for accurate chromosome alignment at metaphase in HeLa cells; the shRNA-mediated knockdown of NDR1 or Fry caused chromosome misalignment on the metaphase plate and delay of mitotic progression. Cotransfection of wild-type but not kinase-dead NDR1 with NDR1 shRNA decreased the number of cells with chromosome misalignment, indicating that the kinase activity of NDR1 is required for accurate chromosome positioning. The kinase activity of NDR1 increased in early mitotic phase and was dependent on Fry and MST2. Chromosome misalignment in MST2-depleted cells was corrected by expression of active NDR1 bearing a mutation that mimics MST2-mediated phosphorylation, but not by expression of a non-phosphorylatable mutant of NDR1. We also provide evidence that Fry binds to microtubules, localizes on the mitotic spindle, acts as a scaffolding protein that binds to both NDR1 and MOB2, and synergistically activates NDR1 with MOB2. These results suggest that the MST2/Fry/MOB2-mediated activation of NDR1 is crucial for the fidelity of chromosome alignment in mitosis.

1368/B526
A CK2-dependent Mechanism for Activation of the JAK-STAT Signaling Pathway.
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The protein kinase CK2 (formerly termed casein kinase-2) controls multiple cellular functions by regulating crucial signaling pathways. However, the regulatory role of CK2 in the JAK-STAT pathway is poorly studied. We report that knockdown of CK2 by small interfering RNA inhibits Oncostatin M (OSM) -induced JAK2 and STAT activation, and expression of SOCS-3, a STAT-3 downstream gene. Inhibition of CK2 by pharmacological inhibitors also blocks OSM-induced activation of JAK2 and STATs and phosphorylation of gp130, the signaling subunit of the OSM receptor. Moreover, endogenous CK2 associates with JAK1 and JAK2, but not with STAT-3, and CK2 phosphorylates JAK2 in vitro. Collectively, our studies identify that CK2 is required for activation of JAK-STAT pathway, and suggest that it may function through interacting with or phosphorylating JAKs.
1369/B527
Atypical Protein Kinase C Regulates TGFβ Dependent Cell Signaling.
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Transforming growth factor beta (TGFβ) family members are involved in many aspects of cellular behavior and aberrant TGFβ signaling has been linked to various human pathological disorders. TGFβ receptor activation can result in the downstream signaling cascades of two distinct pathways, namely through the phosphorylation of Smad2 or the phosphorylation of Par6. We have found that atypical protein kinase C (aPKC) family members play a role in modulating both prongs of the TGFβ pathway. Here we show that PKC iota interacts with TGFβ receptors through the adaptor Par6, and that these proteins localize to the leading edge of migrating cells. Pharmacological inhibition of PKC kinase activity shifts TGFβ receptors into the early endosome and reduces TGFβ receptor degradation. Furthermore, pharmacological inhibition of aPKC isoforms extends TGFβ induced Smad2 phosphorylation. siRNA directed at the aPKC isoforms also extended Smad2 phosphorylation levels providing confirming evidence that aPKCs are negative regulators of TGFβ signaling. Moreover, we have found that phosphorylation of Par6 also increases in the presence of aPKC. aPKC kinase activity as well as association with Par6 were found to be important for Par6 phosphorylation as evidenced by a loss in phospho-Par6 using a kinase deficient mutant of PKCζ (PKCζ-KR) as well as a mutant of Par6 that does not bind aPKC (Par6 K19A). In conclusion, our results indicate that atypical PKC modulates two facets of the TGFβ pathway by negatively regulating TGFβ induced phospo-Smad2 levels and positively regulating phospho-Par6 levels.

1370/B528
Molecular Mechanisms Involved in Thrombin-induced RPE Cell Proliferation.
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The retinal pigment epithelium (RPE) is a monolayer of polygonal cells located between the neural retina and the choroid, and plays an essential role in the maintenance and normal functioning of the retina. Proliferative alterations in RPE cells following the breakdown of the blood-retina barrier (BRB) have been associated with the development of several ocular pathologies, including proliferative vitreoretinopathy (PVR). Under these conditions, RPE is exposed to serum components, among which thrombin has been related with the onset of PVR. The serine protease thrombin activates a family of G protein-coupled receptors (GPCR) named Protease-Activated Receptors (PARs). PAR-1, the best studied receptor in this family, has been shown to increase intracellular IP3 and calcium concentration and to promote the activation of phosphoinositide 3-kinase (PI3K), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) ERK1/2. Although RPE cell proliferation is increased by thrombin stimulation, the molecular mechanisms involved in this outcome are poorly understood. In this work we demonstrated that activation of PAR-1 by thrombin induces the expression and nuclear translocation of cyclin D1, which promotes cell cycle progression. This process is mediated by the activation of transcription factors CREB and NFkB, and the stimulation of c-Fos expression. Our results show that PI3K, PKC and MAPKs ERK 1/2 activation is required for thrombin-induced RPE proliferation. Evidence here provided contributes to the understanding of the mechanisms involved in proliferative eye pathologies, such as PVR. This work was partially supported by Grants IN228203 (PAPIIT, U.N.A.M) and 42640-Q (CONACyT) to A.M.L.C.

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Intracellular Dynamics of Single STAT1 Transcription Factors upon Cytokine Stimulation.
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STAT signalling plays a key role in signal transduction after cytokine stimulation, mainly in cells of the immune system. STAT1 proteins are transported into the nucleus by a carrier-dependent, but also by a carrier-independent mechanism. The mode of transport is connected to the phosphorylation status of STAT1. Hence, nucleo-cytoplasmic shuttling plays an important role in the STAT signalling pathway. We studied the nucleo-cytoplasmic transport of STAT1 before and after activation by cytosolic microinjection of covalently fluorescence labelled STAT1 proteins in HeLa cells. The time course of the subcellular distribution was monitored by confocal microscopy In Vivo over a time period of 60 min. As expected phosphorylated and IFNγ-activated wildtype (wt) STAT1 accumulated in cell nuclei compared to an inactivated truncated (tc) STAT1 mutant and non-activated wt STAT1. Next, we employed high-speed single-molecule microscopy for the analysis of the cytoplasmic and nuclear mobility, the nuclear translocation and intranuclear binding of STAT1 in living cells at 37°C. Both, tc and wt STAT1 showed a balanced distribution of fast, slow and immobile molecules in the cytoplasm. within the nuclei tc STAT1 was significantly more mobile than wt Stat1. IFNγ stimulation induced a shift towards lower mobility of wt Stat within the cytoplasm, and a drastic immobilization within the nuclei. As expected phosphorylated wt Stat1 displayed a similar behaviour. A detailed statistical analysis of single wt STAT1 trajectories clearly revealed numerous confinement events upon IFNγ-activation and allowed for the first time to determine the corresponding distribution of binding times.

1372/B530
Differential Rac Activation by Major and Minor Group Rhinovirus Results in Altered MAPK Activation and Cytokine Release from Human Macrophages.
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Viral respiratory infections often contribute to the pathogenesis of asthma. Human rhinoviruses (HRV) are responsible for the majority of common cold infections and are a leading cause of asthma exacerbation. Major- and minor-group HRV enter cells by binding to the cell surface molecules ICAM-1 and LDL-R that are present on epithelial cells and macrophages. The focus of the resulting viral infection is in bronchial epithelial cells. Despite this narrow tropism, previous studies of HRV infection and the resultant cytokine dysregulation have implicated the predominant immune cells present in the airway, the alveolar macrophages, as playing a role in establishing the proinflammatory environment seen in HRV infection and asthma exacerbation. We demonstrate that the small molecular-weight G-protein Rac1 is differentially activated by the binding of major- and minor-group rhinovirus to macrophages, that MCP-1 release differs between macrophages exposed to the two viruses, and that inhibition of Rac1 attenuates the activation of the stress kinase p38 and the release of MCP-1. Interestingly, RANTES release is not affected by Rac inhibition. We also demonstrate that Rac1 activation does not occur in the epithelial cell lines HeLa and A549 following HRV exposure. Additionally, the related G-proteins cdc42 and H-Ras are not activated in either epithelial cells or macrophages following HRV exposure. This is the first report of a relationship involving macrophages, major- or minor-group HRV exposure, small molecular-weight G-protein activation, and MCP-1 release, suggesting that Rac1 plays a role in establishing the inflammatory microenvironment initiated in the human airway upon exposure to rhinovirus.

1373/B531
Mechanism of Protein Phosphatase 1 Activity Regulation by TIMAP.
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The Ser/Thr protein phosphatase 1 holoenzymes consist of a conserved catalytic subunit (PP1c α, β or γ) and diverse regulatory subunits. PP1 regulatory subunits define substrate specificity, sub-cellular localization and PP1c activity. TIMAP[1-567] is a PP1c regulatory subunit in the myosin phosphatase family restricted to endothelial cells (EC). TIMAP directly associates with the pro-angiogenic 67kDa non-integrin laminin receptor (LAMR1). In living cells, TIMAP/PP1c dephosphorylates LAMR1. but in vitro, TIMAP inhibits PP1c-mediated dephosphorylation of the
nonspecific substrate phosphorylase A. Here we sought to resolve whether TIMAP confers phosphatase specificity towards LAMR1. As expected, immobilized GST-TIMAP[1-567] bound recombinant LAMR1 and PP1cβ with high affinity. LAMR1 was phosphorylated In Vitro by protein kinase C (PKC) and -A (PKA), shown by [32P]ATP labeling in the presence, but not the absence of the recombinant kinase. In the absence of TIMAP LAMR1 was readily dephosphorylated by PP1cβ, whether pre-phosphorylated by PKC or PKA. In the presence of equimolar concentrations of TIMAP/PP1cβ, the phosphatase activity against PKC-prephosphorylated LAMR1 was inhibited by 69 ± 3%. Under identical conditions, the PP1cβ activity toward PKA-prephosphorylated LAMR1 was inhibited by only 15±13% (p<0.02, mean±SEM, n=3 separate experiments). GST alone did not inhibit PP1cβ activity towards PKC- or PKA-prephosphorylated LAMR1. TIMAP[1-567] inhibited the PP1cβ activity against his-LAMR1, whether LAMR1 was pre-associated with TIMAP or not. TIMAP[46-453] also bound PP1cβ and inhibited its activity toward PKC- but much less toward PKA-prephosphorylated his-LAMR1. TIMAP[46-295] neither bound PP1cβ nor inhibited its activity. Hence, binding of PP1cβ to TIMAP inhibits its phosphatase activity against PKC-, but much less against PKA-prephosphorylated LAMR1. It is therefore attractive to postulate that association of PP1cβ with TIMAP allows activity only against very specific substrates, here the PKA- but not the PKC-phosphorylated site of LAMR1. The functional significance of PKC and TIMAP/PP1cβ sensitive phosphorylation sites in LAMR1 in EC will need to be further defined.

1374/B532
Quantitative Analysis of the Adaptor GRB2 Proteome through a Selected Reaction Monitoring (MRM) Assay.
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Adaptor proteins are composed exclusively of interaction domains and binding motifs. The growth factor receptor-bound protein 2 (GRB2) is one such adaptor that modulates cellular responses by directing protein complex formation in signalling networks. Its two Src-Homology 3 (SH3) domains bind proline-rich motifs while its single SH2 domain connects to tyrosine phosphorylated (pYXN) sequences. We have stably expressed a wild-type 3xFlag-tagged form of human GRB2 in human embryonic kidney (293T) cells. We have affinity-precipitated protein complexes and analyzed the components using mass spectrometry (LC/MS/MS). We have identified 104 proteins associated with GRB2 in 293T cells, half of which being novel interactions, and have designed a mass spectrometry-based scheduled MRM (sMRM) assay to quantify their abundance and changes in their phosphorylation state under different conditions. Treatment of cells with a tyrosine phosphatase inhibitor revealed that over half of these interactions are induced by tyrosine phosphorylation, and therefore likely involve binding of the GRB2 SH2 domain with pTyr-containing proteins. Time course (0-100 min) of Epidermal Growth Factor (EGF) stimulation of cells revealed at least three distinct groups of proteins recruited to the GRB2 network: (1) immediate but transient (e.g. SHC1), (2) constitutive (e.g. WASL) and (3) late or absent (e.g. BCR). This study represents the first analysis of the GRB2 proteome at this scale. Furthermore, these data show the high sensitivity and specificity of MRM-based proteomics and its usefulness in cell biology to monitor the abundance and post-translational modifications of large numbers of proteins in a single assay.

1375/B533
Sumoylation of Pellino-1 Protein Involved in Interleukin-1 Receptor/Toll-like Receptor Signaling.
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Pellino-1 protein with E3 ligase activity of its own RING-like domain has been proposed to mediate interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) signaling through the polyubiquitination of interleukin-1 receptor associated kinases (IRAKs). In addition, Pellino-1 protein has been reported to be polyubiquitinated by IRAKs and subsequently degraded. In this
study, we demonstrate that Pellino-1 protein is modified by SUMO-1 (small-ubiquitin-related modifier-1), one of post-translational protein modifiers. Sumoylation assays with Pellino-1 and SUMO-1 expression plasmids clearly revealed that Pellino-1 protein is sumoylated In Vitro and in vivo. Treatment of SENP1 inhibited the sumoylation of Pellino-1 protein and GST-pull down assay as well as yeast two hybrid assay showed that Pellino-1 bound to Sumo-conjugating enzyme, Ubc9. Furthermore, we identified the five lysine residues of Pellino-1 protein which are bound to SUMO-1. These findings strongly suggest the possibility that Pellino-1 protein is subjected to be regulated by SUMO modification as well as polyubiquitination.

**Cell Cycle Controls II (1376 – 1396)**

1376/B534

**Analysis of Changes in DNA Amount and Degree of Chromatin Condensation in Live Cells Using a Novel 488 nm-excitable, Green-emitting Molecular Probe.**

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Ultraviolet (UV)-excited fluorescent live-cell DNA probes (e.g. the Hoechst dyes) have previously been used for live cell cycle analysis and apoptosis detection via flow cytometry and fluorescence microscopy. However, many clinical and research laboratory flow cytometers are equipped with only one or two single-wavelength lasers, typically an argon-ion or solid-state 488 nm laser and a red Helium-Neon or laser diode, emitting in the 633 to 635 nm range. Consequently, we have developed a 488 nm-excitable, green-emitting DNA binding dye suitable for live cell imaging, quantitative cell cycle analysis and enumeration of apoptotic nuclei, as revealed by nuclear condensation. The probe allows for optimization of experimental conditions using conventional fluorescence microscopy, then seamless integration into standard flow cytometry workflows, without the requirement of implementing additional esoteric laser sources. By flow cytometry, nuclear condensation is detected as a population of cells with 50 X enhanced fluorescence relative to their healthy counterparts. In addition, the green-emitting molecular probe can be used without extensive optimization over a wide range of temperatures, buffer conditions and staining intervals to provide high quality data for cell cycle analysis. Protocols for cell cycle analysis and detection of apoptosis have been validated to perform effectively on a variety of platforms, including fluorescence microscopy, flow cytometry and microplate-based cytometry. The described fluorescent probe should prove useful in applications ranging from target identification and validation to small molecule efficacy and toxicity testing.

1377/B535

**14-3-3γ Mediates Cdc25A Proteolysis to Induce S and G2 Arrest After DNA Damage.**

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14-3-3 proteins control a wide variety of cellular processes, such as cell cycle progression, DNA damage checkpoint and apoptosis, through the docking of numerous Ser/Thr-phosphorylated proteins. During DNA damage checkpoint, some subtypes of 14-3-3 (β and ζ isoforms in mammalian cells and Rad24 in fission yeast) binds to checkpoint kinase 1 (Chk1) phosphorylated at Ser345 by ATR and promote the chromatin association of Chk1. Here, we report that γ subtype of 14-3-3 forms a complex with Chk1 in the cells when DNA damage occurs and this binding depends on Chk1 phosphorylation at Ser296 but not at ATR sites (Ser317 and Ser345). Ser296 phosphorylation is catalyzed by Chk1 itself after Chk1 phosphorylation by ATR and then ATR sites appear to be rapidly dephosphorylated on Ser296-phosphorylated Chk1. Although Ser345 phosphorylation is observed at nuclear foci where ATR is considered to be activated, Ser296 phosphorylation occurs more diffusely in the nucleus. The replacement of endogenous Chk1 with
Chk1 mutated at Ser296 to Ala (S296A) abolished G2/M checkpoint in response to UV irradiation, suggesting the importance of Ser296 phosphorylation in DNA damage response. Although Ser296 phosphorylation induces the only marginal change in Chk1 catalytic activity, 14-3-3γ binding to Ser296-phosphorylated Chk1 results in the complex formation between Chk1 and Cdc25A on 14-3-3γ. This ternary complex formation plays a critical role in Cdc25A phosphorylation at Ser76 by Chk1, resulting in Cdc25A degradation after UV-irradiation. Our findings reveal that 14-3-3γ mediates Chk1-induced Cdc25A proteolysis through the complex formation between Chk1 and Cdc25A to induce cell cycle arrest after DNA damage.

1378/B536

Low Power Infrared Laser on Escherichia coli Cultures.

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Applications of low power laser to treat muscular inflammatory processes have increased worldwide due its photothermal effects on tissues. However, data about the adverse effects of laser on DNA are scarce. The aim of this work was to evaluate effects of low power infrared laser on bacterial cultures proficient and deficient on the repair mechanisms of DNA lesions. E. coli AB1157 (wild type), AB1886 (uvrA-), BH20 (fpg-) and BW9091 (xth-) cultures, in exponential growth phase, were irradiated with infrared laser (830nm) at continuous and pulsed (2.5; 250 and 2500Hz) and at different fluencies (1, 4 and 8 J/cm²). After that, aliquots were spread onto Petri dishes with nutritive medium and incubated (37°C, 18 hours). Colony units forming were counted and survival fractions (SF) were determined. Data obtained of SF, at the higher fluency, for AB1157 were (X and SD): 1.34 SD 0.59 (continuous); 1.21 SD 0.49 (2.5Hz); 1.32 SD 0.31 (250Hz); 1.37 SD 0.74 (2500Hz); for AB1886: 0.73 SD 0.17 (continuous); 1.27 SD 0.25 (250Hz); 1.44 SD 0.54 (2500Hz); for BH20: 1.31 SD 0.38 (continuous); 1.02 SD 0.42 (2.5Hz); 1.22 SD 0.12 (250Hz); 1.34 SD 0.20 (2500Hz). These data indicate decreasing of survival of E. coli AB1886 cultures exposed to laser radiation at continuous and pulsed modes, at evaluated frequencies. Results obtained suggest that exposition at low power infrared laser (830nm) could induce lesions in the DNA of E. coli cells whose repair may depend of the gene uvrA-product.

1379/B537

Protein Phosphatase PP6 is Involved in the Repair of DNA Double-stranded Breaks.

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The phosphorylated form at serine-139 of the histone H2A variant H2AX, gamma-H2AX, is a marker for DNA double-stranded breaks (DSBs), and elimination of gamma-H2AX at DNA damage foci is required for DNA damage repair. DSBs are mainly repaired by homologous recombination (HR) and non-homologous end joining (NHEJ). Here we found that endogenous protein phosphatase PP6C forms several distinct trimeric complexes in HeLa cells, and more than six such complexes efficiently dephosphorylates gamma-H2AX in vitro. Depletion of PP6C leads to an increase of gamma-H2AX in vivo, and confers a defect in HR-mediated I-SceI-induced DSBs. Our results suggest that protein phosphatase PP6, along with PP2 and PP4, is required for efficient HR-mediated repair of DSBs.

1380/B538

Platelet Activating Factor Induces a Post-Replication Checkpoint Response at the S/G2 Transition of the Cell Cycle and Blocks Interkinetic Nuclear Migration in Retinal Progenitor Cells.

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The nucleus of proliferating retinal cells migrates back and forth through the neuroblastic layer (NBL) according to the cell cycle phases, an event known as interkinetic nuclear migration (INM). Thus, DNA is duplicated in the innermost margin, while mitosis occurs in the ventricular surface and G1 and G2 phases occur along the nuclear migration pathway. In order to monitor DNA integrity and coordinate proliferation, cells trigger regulatory mechanisms called checkpoints mediated by protein kinases such as CHK1/2, the activation of which occurs upon DNA damage leading to cell cycle arrest. Platelet-activating-factor (PAF), a potent lipid mediator, regulates proliferation in various cells but, its effects upon the cell cycle are still unknown. Since PAF-aceyl-hydrolase, an enzyme complex that inactivates PAF, was shown to be involved in a highly conserved pathway that controls nuclear migration, we wondered whether PAF affects the INM in the developing rat retina. PAF partially inhibited nuclear migration in proliferating cells, pulse-labeled with Bromo-deoxyuridine (BrdU), in the absence of either DNA synthesis blockade or DNA damage and its removal reestablished normal INM. Although the velocity of INM in cells that passed the S/G2 transition was the same in either PAF-treated or control retinal tissue, the reduced number of cell nuclei that reached the outer stratum of the NBL had a heterochromatic pattern of labeling for BrdU, suggesting its incorporation at the end of S phase. Cyclin B1 and phospho-H3 histone levels, markers for G2 and M phase entrance respectively, were lower in BrdU-labeled cells after PAF treatment, suggesting that PAF induced an arrest during S/G2 transition. Pre-treatments with PAF receptor antagonist, p38 or p42/44 MAPK inhibitors, but not with a PKC inhibitor, prevented the blockade of nuclear migration by PAF. PAF promoted CHK1 activation, which in turn was demonstrated to be required for the S/G2 cell cycle arrest. These data showed that PAF induces a novel post-replication checkpoint at the S/G2 phase transition, mediated by CHK1, and independent of either DNA damage or inhibition of DNA replication, thereby resulting in blockade of INM in the developing retina.

1381/B539
**Sumoylation Activity of Ubc9 is Essential for Retinal Stem/Progenitor Cell Proliferation.**
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Objective: In the developing central nervous system (CNS), properly controlled stem/progenitor cell proliferation is crucial for the normal development of each organ with a proper size. Although factors that directly affect the regulation of cell cycle have been relatively well described, the molecular mechanisms which control the size of each region in the developing CNS still remain unclear. We previously reported the essential role of xhmgb3 in the retinal cell proliferation. Here, we further analyzed the molecular mechanisms of proliferation control in the developing retina.

Methods: We performed yeast two-hybrid screen to find out a binding protein to xhmgb3, and identified Ubc9. We investigated the roles of xhmgb3 and Ubc9 in the developing *Xenopus* retina by injecting synthetic mRNAs followed by the measurement of the eye size, immunostaining, biochemistry and *in situ* hybridization. Results: We previously explored molecular targets of transcription factor rax/Rx, which is essential for retinal progenitor proliferation, and found that xhmgb3 functions as one of the downstream factors of rax. We identified Ubc9 as a binding protein to xhmgb3 by yeast two-hybrid screen. Overexpression of xhmgb3 and Ubc9 resulted in enlarged eye size while Ubc9 alone did not. In contrast, loss of function of Ubc9 using dominant negative mutant of Ubc9 resulted in the decreased size of the eye. Conclusions: We found that sumoylation activity of Ubc9 has an essential role in the regulation of retinal stem/progenitor cell proliferation.

1382/B540
**Convergent Evolution of a CDK4/6-Like Cyclin Dependent Kinase in *Chlamydomonas* That Mediates Cell Size Checkpoint Control.**
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Proliferating cells coordinate growth and division through size checkpoints whose underlying mechanisms are not well-understood. The unicellular alga *Chlamydomonas reinhardtii* uses size checkpoints to regulate its multiple fission cell cycle where growth and division are partially uncoupled, and is uniquely suited for dissecting cell size control. The *Chlamydomonas* retinoblastoma (RB) tumor suppressor homolog MAT3, is a cell cycle repressor and key mediator of size control, but it is not known how MAT3 and its conserved target E2F1-DP1, are regulated in response to cell size. Using forward genetic screens we isolated two mutant alleles of a gene designated *CDKG1* whose loss-of-function phenotype is large cells and which functions upstream of MAT3/RB in a linear genetic pathway. *CDKG1* encodes a novel cyclin dependent kinase with a SDSTIRE sequence in place of the canonical PSTAIRE motif and a unique 90 amino acid N-terminal extension. Nonetheless, *CDKG1* was able to complement a budding yeast cdc28-13 temperature sensitive mutation indicating that it retains the core functions of a cell cycle CDK. Yeast two-hybrid screens and In Vitro pull-down assays identified two D-type cyclins, CYCD2 and CYCD3, as binding partners for CDKG1. CDKG1 was also found to bind RB/MAT3 In Vivo and in vitro, and demonstrated MAT3 kinase activity that was stimulated by D cyclins. *CDKG1* mRNA was found to be cell cycle regulated with a peak during S and M phases, and transgenic strains that expressed CDKG1 from a constitutive promoter showed a small-cell phenotype indicating that CDKG1 activity is rate limiting for size checkpoint control. The evolution of CDKG1 in the algal lineage is a remarkable case of convergence with metazoans where the non-essential kinases CDK4/6 and D-type cyclins are used to regulate RB and other pocket proteins. The mechanism by which CDKG1 expression and kinase activity are coordinated during the cell cycle to regulate MAT3/RB and E2F1-DP1 is the subject of ongoing investigation. Supported by ACS RSG-05-196-01-CCG to J.G.U.

1383/B541

**Asymmetric Protein Biomarkers of Asymmetric Self-Renewal Associated With Adult Stem Cells.**

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Adult stem cells (ASCs) drive tissue renewal in adult mammals, and thus they have potential use for several ASCs-based biomedical therapies. Asymmetric self-renewal is a unique characteristic of ASCs that restricts their carcinogenic potential by limiting their numbers. Molecular markers for ASCs, related to their defining asymmetric self-renewal, would be ideal for identifying and monitoring ASCs during clinical applications. Since markers that identify ASCs specifically and universally are unknown, we set out to develop a new method for evaluating mechanisms that regulate adult stem cell regulation by using model murine cell lines that undergo experimentally controlled asymmetric self-renewal, like ASCs. First, we investigated asymmetric self-renewal by the model cell lines using antibodies for the cell cycle-specific proteins cyclin A, E and D. By in situ immunofluorescence analyses, we discovered that, when asymmetrically self-renewing cells were arrested as binucleated cells with cytochalasin D, there were many examples in which only one sister nucleus expressed the tested cyclin. Such asymmetric protein expression in binucleated cells was not detected in cells undergoing symmetric self-renewal. Next, we investigated whether several proteins encoded by asymmetric self-renewal associated (ASRA) genes, recently identified in our lab, also showed asymmetric binucleate expression in asymmetrically self-renewing cells. Here, we report that the transcription regulator histone H2A.Z and the B-cell translocation gene 2 protein (BTG2) show an asymmetric binucleate expression pattern specifically in asymmetrically self-renewing cells. Moreover, examination of H2A.Z and BTG2 in untreated cells in micro-colonies produced by asymmetric self-renewal was consistent with the proteins’ detection being limited to asymmetrically self-renewing ASC-like cells. These studies define novel asymmetric protein biomarkers as a basis for detection of asymmetrically self-renewing cells in complex cell populations. Future studies will evaluate whether these
asymmetric patterns of cell cycle-specific proteins and newly-identified ASRA proteins can be used to detect naturally-occurring ASCs.

**1384/B542**

**Temporal Analysis of Cell Cycle Progression in Immortalized and Primary Cell Line Using Fluorescent Protein Biosensors.**

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Information about cell cycle progression is commonly taken from large numbers of cells at any given point in time, however a more accurate approach to measuring cell cycle progression is to monitor a set number of cells over time. While traditionally this has been difficult, Miyawaki and colleagues recently developed a fluorescence ubiquitination cell cycle indicator (FUCCI), a fluorescent protein-based sensor which allows the progression of cells through the cell cycle to be visualized using fluorescence microscopy. We have used this technology in combination with viral-mediated gene delivery to facilitate highly efficient delivery of these biosensors to a variety of cell types including primary cells. We used this approach to characterize the mean time in which a cell spends in each phase of the cell cycle and how these tightly regulated progressions are affected by various drugs known to disrupt the cell cycle. Moreover, we compare the mean time spent in each phase of the cell cycle between primary and immortalized cell types. The data presented herein describes new temporal details of cell cycle progression.

**1385/B543**

**Increased Efficiency and Speed of Reprogramming of Human Cells into Induced Pluripotent Stem Cells using High-Titer Lentiviral Vectors Encoding Cell Cycle Progression and Survival Genes.**

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Objective: Differentiated cells can be reprogrammed into induced pluripotent stem cells (iPSC) with enforced expression of multiple transcription factors. We aim to improve the reprogramming efficiency using high titer lentiviral vectors encoding additional cell growth and survival regulatory genes. Methods: Lentiviral vectors encoding multiple cell cycle and apoptosis genes in addition to c-Myc, Klf4, Oct4 and Sox2 were constructed and used to generate iPSC from fetal and adult human cells. The iPSC were extensively characterized by immunohistochemical staining and flow cytometry. Results: While human mesenchymal cells can be efficiently transduced and reprogrammed into iPSC using high-titer lentiviral vectors encoding the four known transcription factors, the addition of siRNA suppressing p53 and cell cycle and survival genes including telomerase, CDK4 and BclXL significantly increased the efficiency and speed of iPSC generation. Human iPSC colonies were visualized within a week after lentiviral gene transfer. Conclusions: The protocol for iPSC generation can be improved with high titer lentiviral vectors encoding additional immortalization cellular factors regulating cell cycle progression, senescence and apoptosis. Deletion of the integrated lentiviral genomes using Cre-loxP recombination could improve the safety profile of the reprogrammed iPSC.

**1386/B544**

**Identification of Senescence Genes by Genome-wide shRNA Screen.**

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Senescence has been proposed to act as an antitumor mechanism as well as contribute to the aging process. Understanding the molecular mechanisms of senescence has important implications for tumorigenesis and aging. However, the genetic pathways that activate senescence remain elusive. To gain a better understanding in the molecular mechanisms of senescence, we applied a genome-wide loss-of-function screen using shRNA library to identify
genes whose absence can prevent senescence. In a preliminary screen, we have identified 6 genes, whose deficiency potentially prevents senescence induced by Smurf2. Two of these genes, SUMO2 and catalase have been implicated in senescence regulation. These findings suggest that the genome-wide shRNA screen is an effective approach to identify the genetic components of the senescence pathway.

1387/B545
Male-Germ-Cell-Associated Kinase Regulates the Activity of APC/C-Cdh1 and Accurate Cell Cycle in Prostate Cancer Cells.
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Human Male germ cell Associated Kinase (MAK) is a male specific kinase, mainly expressed in germ cells at and after meiosis. Aberrantly over-expression of MAK is found in prostate cancer cells, and is involved in prostate cancer progression through its roles in androgen signaling. Androgen receptor (AR) is androgen-induced transcriptional factor regulating gene expression essential for prostate cancer cell survival and proliferation. Transcriptionally induced by AR, MAK functions with a feedback mechanism as a co-activator of AR. In addition to AR activation, in this study, we found that MAK also plays an important role in cell cycle regulation. The expression and sub-cellular localization of MAK is dynamic through out the cell cycle: its expression peaks at the G1 phase, and it is localized on centrosomes and mitotic spindle during metaphase and anaphase; on the mid-body during telophase. Over-expression of MAK in LNCaP prostate cancer cell results in centrosome number aberration and multipolar spindle up to 2.5 folds. Whether the mitotic deficiency in the MAK-overexpressed cells leads to genomic instability remains further study. We found that the mechanism underlying the MAK-induced mitotic defect is possibly due to de-regulation of APC/C-Cdh1, which is responsible for accurate mitotic exit and a stable G1 phase. MAK phosphorylates and associates with the APC/C activator CDH1/FZR1, and the activation of ubiquitin ligase APC/C-Cdh1 is inhibited by over-expression of MAK, consequently leading to accumulation of its target proteins such as Aurora A, PLK1 and Cdc20. Our data reveal a novel role of MAK in the control of accurate mitosis, which may contribute to AR-independent prostate carcinogenesis.

1388/B546
Methanol Extract of Hamamelis Japonica Increases P53 Expression in HT1080 Tumor Cell.
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Hamamelis japonica (H. japonica), a deciduous shrub with alternative leaves, is a family of Hamamelidaceae. Hamamelis has been used as a medicinal plant in North America. Activated p53 can induce growth arrest to allow cells to repair the damage or apoptosis. Since some molecules can activate wild-type p53 by disrupt Mdm2-p53 binding or inhibit Mdm2 E3 ubiquitin ligase, we investigated the effect of the methanol extract of H. japonica on p53 activity. The expression of wild-type p53 in HT1080 human fibrosarcoma cells was increased in a dose-dependent manner following 10-hr treatment of up to 50 ug/ml of the H. japonica extract. Furthermore, G2 arrest was induced with increasing concentrations of the extract. However, the mRNA level of p53 was suppressed; it may caused by negative feedback. Isolation of a molecule(s) from the extract responsible for p53 activation would pave the way for the development of a new generation of growth arrest inducing anticancer drugs.

1389/B547
Insights into the Retinoblastoma (RB) Tumor Suppressor Pathway Derived from Proteomics and Phosphopeptide Mapping in the Unicellular Model Organism Chlamydomonas reinhardtii.
Retinoblastoma (RB) related proteins are conserved cell cycle regulators that control S phase entry in many eukaryotes, including the green alga *Chlamydomonas reinhardtii*. *Chlamydomonas* cells lacking the RB homolog MAT3 exhibit inappropriate cell cycle progression resulting in a small-cell phenotype. Mutations in *E2F1* and *DP1* suppress the small-cell phenotype of *mat3* null strains, indicating conservation of the RB pathway in *Chlamydomonas*. This finding was confirmed by yeast two-hybrid and co-immunoprecipitation (co-IP) assays where we found DP1 and E2F1 interact as a heterodimer and that RB/MAT3 interacts with both DP1 and E2F1. Surprisingly, periodic transcription of cell cycle regulated genes was not altered in RB pathway mutants, a finding that suggested a post-transcriptional role for RB/MAT3 that has prompted further investigation of MAT3/RB protein complexes. The relative abundance of MAT3 remained unchanged throughout the cell cycle as indicated by quantitative Western blotting. In contrast, DP1 was found to increase significantly during S phase and mitosis. Interestingly, the relative amount of association between DP1 and MAT3 did not change at any time during the cell cycle, suggesting that dissociation of MAT3 from E2F1-DP1 heterodimers is not required for cell cycle progression. In accordance with these data, immunofluorescence experiments showed that MAT3 and DP1 remained in the nucleus throughout the cell cycle. Mass spectrometry of immunopurified MAT3/RB complexes is being used to find additional interacting proteins. Besides finding E2F1 and DP1 in our IP pellets, we identified chromatin remodeling proteins and several novel proteins whose function in cell cycle control is under investigation. In parallel, we are using naturally synchronized cultures grown in a diurnal light:dark cycle to examine cell cycle phosphorylation of MAT3/RB that has around 12 well-defined tryptic phosphopeptides as assayed by two-dimensional chromatography. Work is in progress to determine the cell cycle kinetics of MAT3 phosphorylation and the kinases that control its activity.

1390/B548

**Tylophorine Arrests Carcinoma Cells at G1 Phase and Retards S Phase Progression by Inhibiting DNA Synthesis.**

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We have revealed by asynchronizing and synchronizing approaches that tylophorine not only retards the S phase progression but also dominantly arrests the cells at G1 phase in HepG2, HONE-1, and NUGC-3 carcinoma cells. Moreover, tylophorine treatment results in down regulated cyclin A2 expression and overexpressed cyclin A2 rescues the G1 arrest by tylophorine. We further dissected the inhibitory effect of tylophorine on cell replication and DNA synthesis by BrdU pulse-chase and incorporation experiments. Carcinoma cells were treated with BrdU for 24 hr; ~25%-45% of cells were labeled with BrdU and then the BrdU-labeled cells were chased. With DMSO or dehydro-tylophorine treatment, the proportion of BrdU-labeled cells decreased ~70% in HepG2 cells, ~70%-80% in HONE-1 cells, and ~60%-70% in NUGC-3 cells after 48 hr. In contrast, after 24-hr chase, with tylophorine treatment, the BrdU-labeled cell population showed no significant decrease and after 48 hr chase, decreased only ~5%--20%. Moreover, tylophorine, 2μM, strongly inhibited the BrdU incorporation by the carcinoma cells, as revealed by the relatively less BrdU incorporated into 2N and 4N DNA content. Therefore tylophorine strongly inhibited the growth and DNA synthesis of HepG2, HONE-1, and NUGC-3 cells.

1391/B549

**Does Diepoxybutane (DEB) Increase Cancer Stem Cells and Allow Resistance to Antitumor Therapy?**

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1,3-butadiene (BD) has been deemed as an environmental pollutant and is also a volatile gas used in the production of rubber, plastics, insulation, acrylics, and other polymers. Its ubiquitous occurrence in environmental and industrial areas has been shown to increase the risks of respiratory illnesses. In addition, BD is a known mutagen and human carcinogen, and possesses multi-organ systems toxicity that includes bone marrow depletion, spleen, and thymus atrophy. After entering the body, BD is metabolized to its most toxic metabolite, Diepoxybutane (DEB). Recent studies suggest that some environmental pollutants may increase the stem cell population in several human cancers and that these cancer stem cells can acquire resistance to anticancer drug treatments. In order to elucidate the cellular and molecular mechanisms mediating the acquired resistance to carcinogens in cells, we investigated the effect of DEB on the prevalence of cancer stem cells among the human prostate cancer cell line DU145 utilizing Immunofluorescence and Western blot analysis. We observed that DEB (1µM) increases the incidence of cancer stem cells within the DU145 cell population. By employing specific cell pathway inhibitors, we determined the role played by various cell survival pathways during the DEB-induced prevalence of cancer stem cells in DU145 cells.

1392/B550
TREM2 suppresses the growth of colorectal carcinoma cells.
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TREM (triggering receptor expressed on myeloid cells) is a cell surface receptor that participates in diverse cell processes, including inflammation, bone homeostasis, neurological development and coagulation. TREM2 is involved in macrophage inflammation, microglial phagocytosis and it regulates osteoclast development, but its involvement in cancer cell biology is unknown yet. To determine the involvement of TREM2 in the growth of colorectal carcinoma cells, we overexpressed TREM2 in MC38 colorectal carcinoma cells transiently. TREM2 suppressed the proliferation of colorectal carcinoma cells, and TREM2 expression levels correlate with proliferative activity. The proliferation was also related with cell cycle, which consequently led to regulate the expression of cell cycle regulatory molecules. Furthermore, TREM2 downregulated the activation of colorectal carcinoma-specific signaling molecules and transcription factors. These results suggest that TREM2 suppresses the tumorigenecity of colon carcinoma cells.

1393/B551
Estrogen Receptor Regulates the Expression of E2F1 to Mediate Tamoxifen Resistance.
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Anti-estrogen resistance often develops with prolonged hormone therapies such as tamoxifen treatment, and it is currently a major problem in the treatment of breast cancer. Understanding the mechanism of anti-estrogen resistance is important for the development of more targeted therapies. There are two forms of anti-estrogen resistance: de novo resistance and acquired resistance. To mimic acquired resistance, we have established a tamoxifen-resistant cell line (MCF-7TamR) by treating parental MCF-7 cells with 10-8 M tamoxifen over a period of six months to select for cells with the resistant phenotype. Characterization of the MCF-7TamR cells under normal, hormone-deprived, or tamoxifen-treated conditions suggests that these cells continue to grow in the presence of tamoxifen and that more of them enter the S phase compared to parental MCF-7 cells, which confirms the resistant phenotype of the cells. Consistent with the growth results, analysis at the molecular level indicated that tamoxifen-resistant cells expressed higher levels of cell cycle genes, including those of cyclin E1, cdk2 and E2F1. ICI-mediated degradation of the estrogen receptor reduced the cell proliferation and E2F1 expression of the tamoxifen-resistant cell line, suggesting that tamoxifen resistance and E2F1 expression are in part dependent on the receptor. Additional studies are necessary to dissect the mechanism of tamoxifen resistance and determine the role of ER is this process. Results from this project will
provide valuable information for future therapeutics and may improve the success of current drug therapies.

1394/B552

**A Systematic Analysis of PARP Function.**

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Poly(ADP-ribose) (pADPr) is a macromolecular post translational modification essential for multicellular life. pADPr is synthesized by a family of 17 poly(ADP-ribose) polymerase (PARP) proteins that are largely uncharacterized. Multiple small molecule inhibitors of PARP-1 are currently in clinical trials for treatment of breast and ovarian cancers with genetic lesions in BRCA1/2. Given the pharmaceutical interest in developing PARP inhibitors as potential therapies for human disease, it is important to understand the molecular mechanism of pADPr function and identify functions for the PARP family. pADPr is required for many cellular processes including cell division, chromatin structure regulation, and transcriptional regulation and in cell stress responses such as DNA damage repair and viral infection. The polymer is found throughout the cell and has cell cycle dependent localization patterns. It is therefore necessary to determine when and where specific PARPs function to polymerize functionally relevant pADPr polymer. In order to identify new functions for pADPr and PARPs, we have taken a PARP-family wide approach to identify localization patterns throughout the cell cycle and identify knockdown phenotypes using GFP-PARP fusion, PARP specific antibody, and multiple siRNA libraries we have generated. From these studies we have identified new localization patterns for PARPs and identified new functions for the PARP family of proteins. Our findings have important implications for our understanding of the mechanism of pADPr and PARP function.

1395/B553

**Use of Live Cell Imaging to Construct Stem Cell Lineage Histories and Identify Quiescent Cells.**

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Objective: Regenerative medicine along with stem cell biology requires an understanding in population heterogeneity, including the divisional status of cell populations. Quiescent cells (for which no definitive markers exist) may be an effective cell type in regenerative medicine because of their potential to actively proliferate, giving rise to multipotent progeny. Muscle-derived stem cells (MDSC) are used as a model in order to identify sub-populations focusing on cell lineage and their associated behavioral parameters. Methods: MDSCs were labeled with 5-chloromethylfluorescein diacetate (CMFDA), which decreases in concentration upon subsequent rounds of cellular division. FACS was used to sort the MDSC population into non-dividing (CMFDA positive) and dividing (CMFDA negative) classes. A unique automated cell imaging system was used to capture time-lapsed images in order to establish cell lineage history and analyze motility and morphology. Results: Cellular tracking demonstrated the presence of quiescent cells in CMFDA-sorted populations. A positive correlation was observed between the length of the cell cycle and cellular area. Non-dividing cells were significantly larger than dividing cells (p < 0.05). The rate of cell cycle division also correlated with velocity, as actively dividing cells showed faster cellular velocity compared to non-dividing cells. Conclusion: The use of live cell imaging identified actively dividing and non-dividing cells within a MDSC population. Significant differences were observed between dividing and non-dividing classes. Since the quiescent cells may be critical to the quality of the population, methods to assess this population are valuable tools needed to evaluate the quality of stem cells used for transplantation. Identifying a distinct class of cells will allow for a better understanding of MDSC activity, and allow for the cells with the greatest potential to be used in regenerative medicine.
In Saccharomyces cerevisiae and other eukaryotes, inositol-containing lipids and inositol phosphates are an important component of the phosphatidylinositol signaling pathway. When an inositol-requiring mutant (MC6A) of Saccharomyces is starved for inositol, it undergoes drastic changes in cell metabolism that results in a rapid loss of viability known as inositolless death. In this study, changes in the transcription of cyclin CLB2 were examined to determine if there are changes in the regulation of cell division during in inositolless death. The CLB2 gene codes for a B-type cyclin involved in cell cycle progression. It activates Cdc28p to promote the transition from G2 to M phase and accumulates during G2 and M. It then is targeted via a destruction box motif for ubiquitin-mediated degradation by the proteasome. The levels of mRNAs transcribed from the CLB2 gene were examined using RT-PCR to determine if they declined during inositolless death. Three hours after the start of inositolless death the levels of CLB2 RNAs declined 25.5% when compared to these RNAs from control cells. within 6 hours this level had dropped further to 75.8%. CLB2 gene products should decline because of this reduction in CLB2 RNAs. This should stop the transition from G2 to M phase during cell division. To test this idea, cells from the cultures from which the RNAs were extracted were examined by Differential Interference Contrast (DIC) Fluorescence Microscopy after DAPI staining for changes in the staining of nuclei and budding. DAPI staining of 24h inositol-starved cells indicated the absence of distinct nuclei and a decrease in DNA content. Mitotic spindles also were not visible. In addition, after 3 h there was a 43% decline in budding index, however, after 24 h there was a 3.5-fold increase in the budding index when the starved cells were compared to control cells grown on inositol. This increase in the budding index and lack of mitotic spindles is consistent with what occurs in null CLB2 mutants. The inositol-requiring mutant when starved for inositol may mimic the phenotype of null CLB2 mutants because transcription of the gene is blocked during inositolless death.

Meiosis and Mitosis II (1397 – 1422)

Spindle Motor-Boating in Woods Hole or Probing the Physical Properties of Biological Gradients.

The localized generation of GTP-bound Ran by its chromatin-associated guanine exchange factor (GEF) RCC1 creates a molecular gradient that promotes microtubule polymerization during spindle assembly in mitosis and meiosis. Since the binding of RanGTP to import receptors induces the release of spindle assembly factors (SAFs) from importins, the RanGTP gradient is also translated into a secondary SAF gradient. In interphase, the nuclear envelope limits diffusion of RanGTP and SAFs; however, in mitosis the simplest prediction is that these gradients are defined by diffusion from the surface of the DNA. However, it is also possible that the properties of each gradient are dictated by the presence of a local diffusion-limiting environment rather than by a pure diffusion-based mechanism. To date, these possibilities have not been experimentally tested. To this end, we utilized high-resolution, time-lapse imaging of well characterized FRET probes in Xenopus egg extracts to monitor the response of each gradient to experimentally introduced cytoplasmic flow or to perturbation of the rheological properties of the cytoplasm. We employed what we have deemed the spindle “motor-boating” assay to examine the effects of flow. In brief, bent microneedles were used to skewer spindles, effectively holding them at a fixed position and flow of the surrounding extract was generated by moving the microscope stage. Interestingly, under identical flow conditions, the dimensions of the RanGTP gradient were largely unaffected whereas the SAF gradient became diminished over time. The SAF gradient, but not
the RanGTP gradient, was also sensitive to treatment with the enzyme amylase, which depolymerizes glycogen - a major component of the extract. Taken together, these data indicate that the RanGTP gradient and its resultant SAF gradient have distinct biophysical properties that may be critical in spatially directing microtubule polymerization. Understanding how the dimensions of molecular gradients are established in the environments which they occupy has significant implications in the spatial organization of numerous biological processes at both a cellular and organismal level.

1398/B556
Cdk1 Phosphorylates CLASP2 During Mitosis.
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In the present work we aim to understand how CLASPs are regulated during mitosis. During cell migration in interphase CLASP2 affinity to microtubules (MTs) is negatively regulated through phosphorylation by GSK3β. This phosphorylation appears also to regulate the interaction of CLASP2 with EB1 and IQGAP1. We found that during mitosis, CLASP2, but not CLASP1, is heavily phosphorylated, resulting in a significant band shift on SDS-PAGE. CLASP2 deletion mutant analysis showed that the phosphorylated amino-acids are mainly concentrated in the C terminal of the protein, which regulates kinetochore targeting and binding to several molecular partners, such as CLIP-170. The observed band shift was significantly reduced after inhibition of Cdk1 by the small molecule RO-3306, suggesting that Cdk1 is required for CLASP2 phosphorylation. In Vitro kinase assays with purified Cdk1/Cyclin B showed that Cdk1 directly phosphorylates CLASP2. Similarly, CLASP2 mitotic phosphorylation at the C terminal can be mimicked in Xenopus extracts and the shift is reduced when Cdk1 activity is inhibited. Furthermore, Cdk1 inhibition did not perturb normal CLASP2 localization to kinetochores, spindle or centrosomes during mitosis. Overall, our data indicates that CLASP2 is phosphorylated during mitosis by Cdk1, possibly at multiple residues. We are currently determining the functional implications of CLASP2 phosphorylation during mitosis.

1399/B557
GSK3 Regulates Mitotic Chromosomal Alignment through CRMP4.
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Chromosomal alignment and segregation is an important well-controlled step in mitosis. This process is largely regulated by the mitotic spindle, in which microtubules and microtubule binding proteins capture condensed chromosomes by their kinetochores and direct them to the metaphasic plate. Much effort has been put into identifying the molecular mechanisms responsible for regulating chromosomal alignment and segregation. Glycogen Synthase Kinase 3 (GSK3) is a serine/threonine kinase originally identified as a kinase involved with glycogen metabolism. GSK3 has now been implicated in diverse range of cellular functions including the regulation of mitotic spindle dynamics and chromosomal alignment. Although the importance of GSK3 as a mitotic kinase has been recognized, the physiological substrates that mediate the GSK3-dependent effects during mitosis have yet to be identified. In this study, we investigate the role of Collapsin Response Mediator Protein 4 (CRMP4), a known physiological substrate of GSK3, in mitosis. We identify CRMP4 as important regulator of chromosomal alignment during mitosis in HeLa cells. We show that CRMP4 localizes to spindle microtubules and demonstrate that CRMP4 loss of function leads to defects in chromosomal alignment during mitosis. We provide evidence that CRMP4 is phosphorylated during mitosis in a GSK3-dependent manner and demonstrate that CRMP4 phosphorylation by GSK3 regulates chromosomal alignment.
These findings identify CRMP4 as a downstream regulator of GSK3-dependent chromosomal alignment during mitosis.

1400/B558
Mitotic Spindle Architecture is Modulated by MAP4.
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An important challenge in cell biology is to understand how the combination of molecular motors and non-motile microtubule associated proteins (MAPs) contribute to bipolar spindle assembly and cooperate to govern its steady-state architecture. MAP4 is the major structural MAP in non-neuronal cells, and it has been shown that its overexpression leads to the formation of monopolar spindles (Holmfeldt P, et al, JCS, 2003). However, the precise function of this protein in mitosis is unknown. Using an siRNA-based approach, we show that MAP4 is essential to build a mitotic spindle with normal morphology in prometaphase. Many spindles, in MAP4-depleted cells, lose their normal elliptical shape, appearing bent or diamond-shaped. Such spindles are longer and narrower when compared to control cells. Surprisingly, these spindles are unable to correctly align their chromosomes on a metaphase plate, resulting in a thicker and smaller plate and leading to a mitotic delay. We have found however that the stability of the attachments of microtubules to kinetochores are not affected by MAP4 depletion, suggesting that MAP4 affects chromosome alignment by an alternative mechanism. Based on these observations, we can conclude that MAP4 is a major regulator of spindle morphology and is critical for the accurate alignment of the metaphase plate. We are currently investigating the molecular mechanism by which MAP4 organizes mitotic spindle microtubules and how it cooperates with other motors and MAPs to accomplish its function.

1401/B559
A Previously Uncharacterized Population of Mad1 is Recruited to Metaphase Kinetochores after Taxol Treatment.
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The spindle assembly checkpoint (SAC) ensures anaphase onset does not occur until all chromosomes have been properly bioriented within the spindle. It has been proposed that the SAC monitors two critical inputs: 1) kinetochore-microtubule attachment and 2) tension. Specific signaling molecules have been hypothesized to mediate SAC signaling in response to the state of each of these inputs. For example, a complex of the checkpoint proteins Mad1 and Mad2 is conventionally viewed as an attachment-sensing signaling component since it is highly enriched at unattached kinetochores but difficult to detect at tensionless metaphase kinetochores following treatment with the microtubule stabilizing drug taxol. Pines and co-workers have shown that treating metaphase cells with 10 μM taxol rapidly inhibits APC/C-dependent degradation of Cyclin B. By implementing a more sensitive method to examine kinetochore-bound Mad1-YFP in Ptk cells we found that a population of Mad1 is recruited to most metaphase kinetochores in response to 10 μM taxol treatment. We have recently postulated that another input to the SAC is intrakinetochore stretch and that it acts upstream of kinetochore-bound Mad1 and Mad2. Interestingly, the taxol treatment that led to elevated Mad1 levels at metaphase kinetochores has been shown to have no effect on microtubule attachment although it reduces intrakinetochore stretch. Thus, we have identified a previously uncharacterized population of Mad1 that is recruited to tensionless kinetochores that we hypothesize is reflective of loss of intrakinetochore stretch rather than microtubule attachment.

1402/B560
Assembly of the Mitotic Checkpoint Complex (MCC).
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During mitosis, the mitotic checkpoint prevents premature anaphase onset by monitoring kinetochore-spindle microtubule interactions, ensuring that all chromosomes achieve bipolar attachment and are under tension at the metaphase plate. At the molecular level, this is achieved by inhibiting the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase whose activity is critical for the metaphase-to-anaphase transition and mitotic exit. The In Vivo APC/C inhibitor was proposed to be the Mitotic Checkpoint Complex (MCC), consisting of BubR1/Mad3, Bub3, Mad2, and Cdc20, but there are reports arguing against the presence of MCC in cells. We have found that MCC assembly is subject to cell cycle regulation. The level of Cdc20 and Mad2 associated with the cell cycle-independent BubR1-Bub3 subcomplex increases abruptly during mitosis, and is dependent upon an activated checkpoint. However, forced expression of a Mad2 mutant primarily adopting closed conformation (C-Mad2) induced the assembly of MCC in G1/S cells. This suggests the intracellular concentration of C-Mad2 as a major determinant for MCC assembly, and is consistent with the idea that unattached kinetochores catalyze the conformational change of Mad2 to produce more C-Mad2 molecules in mitotic cells. To further dissect the interactions between MCC subunits during assembly, we have performed a series of In Vitro binding experiments using purified recombinant proteins. An N-terminal fragment of BubR1 (1-371) is sufficient to form a complex with Cdc20 and Mad2 in vitro. Despite earlier data suggesting that Cdc20 functions as a bridging subunit to link BubR1 and Mad2 together in the MCC, we detected direct interaction between BubR1 (1-371) and Mad2. In agreement with In Vitro data, Mad2 mutants deficient in binding Cdc20 were found to be in complex with BubR1 (1-371) in vivo. Future experiments will address the physiological relevance of the BubR1 (1-371)-Mad2 interaction in modulating MCC assembly and APC/C inhibition.

1403/B561

Covalent Conjugation of Nuclear Mitotic Apparatus Protein by SUMO-1 and its Role during Mitosis.

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Covalent conjugations of proteins with Small ubiquitin-related modifier 1 (SUMO-1) plays a critical role in a variety of cellular function including cell cycle control, replication as well as transcriptional regulation. Nuclear mitotic apparatus protein (NuMA) is an essential component in the formation and maintenance of mitotic spindle poles, which is localized to nucleoplasm in interphase and subsequently recruited to spindle poles during mitosis. Here we show that NuMA is a target for covalent conjugation by SUMO-1. Either knockdown of Ubc9, E2 conjugating enzyme in the SUMOylation cascade, or forced expression of SENP1, SUMO-1 deconjugating enzyme, resulted in impairment of proper localization of endogenous NuMA to mitotic spindle poles. We found one of major acceptor lysine residue of NuMA for SUMO-1 conjugation in the C-terminus which is important for its association with mitotic microtubules. The SUMOylation deficient mutant of NuMA induced multiple spindle formation than wild-type NuMA during mitosis. Taken together, the dynamic changes of NuMA through SUMO-modification might contribute to mitotic segregation of chromatin.

1404/B562

Dimeric Centromere Protein E (CENP-E) Promotes Microtubule-elongation at the Plus-ends of Microtubules.

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Centromere protein CENP-E is a dimeric kinesin (Kinesin-7 family) with critical roles in mitosis including establishment of microtubule (MT)-chromosome linkage and processive movement of monooriented chromosomes on MTs for proper alignment at metaphase. Fluorescence microscopy studies were performed to test the hypothesis that CENP-E promotes MT-elongation at the MT plus-ends. CENP-E constructs were engineered, expressed, and purified which yielded dimeric and monomeric motor proteins. The results show that dimeric CENP-E promotes plus-
end directed MT gliding at 11 ± 0.005 nm/sec (n=173 MTs). Real-time microscopy assays were performed to image CENP-E promoted elongation of GMPcPP-stabilized polarity marked FITC MTs. The results revealed that out of the 270 polarity marked MTs examined, 164 MTs (60%) exhibited CENP-E promoted MT plus-end extension by GTP-tubulin (1.48 ± 0.37 µm/30 min; n=200 MTs) in the presence of MgATP. In contrast, dimeric Kinesin-1, dimeric Eg5, and CENP-E in the presence of AMPPNP did not show this pronounced MT elongation. These results suggest that CENP-E as part of its function for chromosome kinetochore attachment to MTs plays a direct role in kinetochore MT extension during congression. Supported by NIH GM54141 to Susan P. Gilbert.

1405/B563

Spindle Length Stability As Mediated by Antagonistic Motor Forces.
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During mitosis, the metaphase spindle provides a stable yet dynamic mechanical structure that is required to correctly mediate faithful chromosome segregation. In the budding yeast mitotic spindle, chromatin stretching forces act as a critical mechanical element that is required to maintain a stable mitotic spindle. In addition, previous work has shown that microtubule dynamics are regulated by chromatin stretching tension both In Vitro and in vivo. Therefore, a key question is how chromatin stretching can contribute to the mechanical stability of the mitotic spindle, while still allowing for proper length regulation of microtubules in the spindle. To address this question, we developed a force balance simulation for budding yeast, which predicts that tension-dependent kinetochore microtubule dynamics give rise to spindle length oscillations when spindles lack minus-end directed molecular motors. In contrast, metaphase spindle length oscillations are minimized by the addition of simulated minus-end directed molecular motors that oppose plus-end directed motors to limit large fluctuations in chromatin stretching forces at kinetochore microtubule plus-ends. Experimentally, we find that spindles lacking the minus-end directed motor Kar3 have spindle length oscillations, an effect that is mitigated by stabilization of kinetochore microtubule plus-ends via a microtubule-stabilizing benomyl treatment. In summary, these results suggest that minus-end directed molecular motors act to limit large fluctuations in chromatin stretching forces in the yeast mitotic spindle, thus promoting stable mitotic spindle lengths.

1406/B564

Involvement of the Mitotic Exit Network in Metaphase Spindle Positioning.
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In budding yeast, the mitotic spindle is functionally and structurally asymmetric, in the sense that the two spindle poles and their associated asters are functionally distinct, recruit distinct regulators and are morphologically different. During metaphase, this asymmetry is crucial to the proper alignment of the spindle with the division axis of the cell. In this case, the microtubule-associated and EB1 Bim1 cargo protein Kar9 decorates only the old spindle pole body (SPB) and the astral microtubules (aMTs) emanating from it. There, Kar9 recruits the actin-dependent motor protein Myo2, a type V myosin, to microtubule tips. In turn, Myo2, mediates the movement of these astral microtubules along actin cables towards and into the bud, and thereby promotes the migration of the old SPB towards the future daughter cell. The new SPB passively orients then towards the mother cell, leading to the alignment of the spindle with the mother-bud axis. Cells where Kar9 localizes symmetrically to spindle poles and asters fail to stably orient their spindle. Therefore, we investigate how the asymmetric localization of Kar9 is established and maintained. Genetic evidence prompted us to test whether the Mitotic Exit Network (MEN) was required for Kar9 asymmetry in metaphase. The MEN is a signaling network that has been described for triggering mitotic exit in telophase in response to spindle alignment. Remarkably, cells carrying temperature-sensitive alleles of TEM1, CDC15, NUD1 as well as cells lacking the downstream kinase Dbf2 failed to properly establish Kar9 asymmetry on the metaphase spindle.

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To explain how the MEN pathway promotes Kar9 asymmetry, we present data indicating that Kar9 is one but not the only relevant substrate of Dbf2 during spindle alignment. Furthermore, our data indicate that during metaphase the MEN pathway helps dissociate Kar9 from the new aster, indicating that it is active mostly on the new SPB. Next, we wondered whether the asymmetric localization of MEN proteins themselves was required for the asymmetry of Kar9. So far, all mutants affecting MEN asymmetry affected Kar9 distribution only mildly. Altogether, our data suggest a novel role for the MEN in spindle positioning during metaphase.

1407/B565
Loss of Intrakinetochore Stretch of the Ndc80 Complex May Signal to the Spindle Assembly Checkpoint (SAC) through Mad1/Mad2.
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The spindle assembly checkpoint (SAC) is a signaling pathway that normally delays anaphase onset until all chromosomes have achieved metaphase alignment. at these aligned kinetochores, the Ndc80 complex of the core microtubule (MT) attachment site becomes moved (intrakinetochore stretch) ~10-15 nm away from the inner kinetochore. There is recent evidence that recruitment or retention of the SAC protein complex Mad1/Mad2 to kinetochores is controlled by intrakinetochore stretch and depends on the calponin homology domain of Ndc80/hsHec1. To test this model, we depleted synchronized HeLa cells of Spindly, a protein required for normal dynein-dependent stripping of Mad1/Mad2 from metaphase aligned kinetochores. When control cells were in late metaphase with very low levels of kinetochore Mad1, aligned chromosomes in Spindly depleted cells exhibited low intrakinetochore stretch and high levels of kinetochore Mad1. The average separation between an antibody that bound Mad1 close to its Mad2 binding site, and an antibody (9G3) that binds hsHec1 next to the calponin homology domain was ~ 3 nm as measured by K-SHREC (kinetochore-fluorescent speckle high resolution co-localization). Spindly knockdown led to a 1-2 hour of mitotic arrest during which kinetochores on aligned chromosomes gradually developed normal intrakinetochore stretch, lost kinetochore Mad1 and eventually entered anaphase. Thus, satisfaction of the SAC in HeLa cells correlates with an increase in intrakinetochore stretch concomitant with the loss of a previously uncharacterized population of Mad1 that is proximal to the MT binding domain of Ndc80. We hypothesize that induction of intrakinetochore stretch satisfies the SAC, in part, by positioning the Ndc80 complex so that it cannot efficiently recruit/retain this population of Mad1/Mad2 at the kinetochore.

1408/B566
Subcellular Localization of the Cdk1/Cyclin B Complex Controls Chromatid Segregation.
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The separation of sister chromatids in anaphase is mediated by separase, a protease that cleaves a subunit of the cohesin complex. Separase can be inhibited by securin and Cdk1/cyclin B. Both securin and cyclin B are degraded during the metaphase-anaphase transition. However, cells depleted of securin still undergo timely separase activation and chromatid separation. Expression of non-degradable cyclin B arrests cells in mitosis but does not block separation of sister chromatids. Here we show that both Cdk1 and cyclin B are localized on chromosomes in prometaphase and metaphase, but disappear from chromosomes shortly before they segregate. To better understand the relationship between removal of Cdk1/cyclin B from chromosomes and chromatid segregation, we treated mitotic cells with the Cdk inhibitor Flavopiridol. Flavopiridol consistently induced most morphological changes of normal mitotic exit. However, chromatid segregation was observed only when cells were treated in metaphase, and not in prometaphase. Metaphase cells treated with Flavopiridol readily cleared Cdk1 and cyclin B from chromosomes, whereas prometaphase cells were less apt to do so. Importantly, only cells that cleared Cdk1/cyclin B from chromosomes were able to segregate sister chromatids. This finding suggests
that removal of Cdk1/cyclin B from chromosomes may be required for cleavage of cohesin. The mechanism underlying the removal of Cdk1/cyclin B from chromosomes likely depends on Cdk1 and not cyclin B, because an N-terminal fragment of cyclin B that does not bind Cdk1 is not removed from chromosomes when chromatids segregate. Triggering the mitotic spindle checkpoint with taxol resulted in retention of Cdk1/cyclin B on chromosomes and completely blocked chromatid segregation in response to Cdk inhibition. We propose that shifting the subcellular localization of Cdk1/cyclinB away from chromosomes plays a key role in separase activation and anaphase onset. In addition, this mechanism may explain why chromatid separation occurs ahead of other events of mitotic exit.

1409/B567
Polarized Myosin Produces Unequal Size Daughters during Asymmetric Cell Division.
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Asymmetric cell divisions produce two different sized daughter cells that ultimately have distinct fates. The best studied examples of asymmetric cell division arise from the initial displacement of the mitotic spindle towards one side of the cell through dynein-dependent pulling forces at the cortex. However, another type of asymmetric division starts with a centered mitotic spindle but then the cleavage shifts towards one side through a poorly understood mechanism. Here, we show that both types of asymmetric cell division occur in the C. elegans Q neuroblast lineage. for the asymmetric division with centered spindles, we find higher levels of myosin on the side of what will become the smaller daughter and show that this unequal distribution is under the control of the MELK family kinase, PIG-1. We propose that asymmetric cortical tension, produced by unequal myosin-based contractile forces and perhaps other factors, can produce different sized daughters during some types of asymmetry cell divisions.

1410/B568
Human Fidgetin Proteins Regulate Anaphase A.
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Fidgetin is a member of the AAA (ATPases Associated with various cellular Activities) protein superfamily which has been proposed to function as a microtubule severing enzyme based on its sequence similarity to Katanin and Spastin. Mammals also contain two additional Fidgetin-like proteins (FIGL-1 and FIGL-2) whose functions remain unknown. Recently we found that Drosophila Fidgetin contributes to anaphase a by stimulating poleward tubulin flux. Here, we examine the mitotic functions of human Fidgetin and Fidgetin-like proteins. We have found that, similarly to its counterpart in Drosophila, human Fidgetin localizes to mitotic centrosomes and its depletion by siRNA significantly reduces the rate of anaphase a (32% decrease relative to control). Depletion of FIGL-1 induces a similar attenuation of anaphase A. Interestingly, co-expression of these proteins in U2OS cells destroys cellular microtubules while their expression individually does not. We are currently investigating whether 1) these proteins alter poleward chromosome movements by suppressing poleward tubulin flux, Pacman, or some combination thereof and 2) whether they work in concert to sever microtubules. Our current working hypothesis is that human Fidgetin proteins sever microtubules from centrosomes thereby creating free minus-ends that serve as a substrate for kinesin-13 driven poleward flux.

1411/B569
UV Microbeam Irradiation of Chromosomal Spindle Fibres in Mesostoma Spermatocytes.
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Mesostoma spermatocytes have 3 bivalents approximately 30-40μm in length, each bipolarly oriented. Mesostoma spermatocytes do not have a defined prometaphase or metaphase because each bivalent’s kinetochore oscillates from the end of prophase until the start of anaphase: each kinetochore regularly moves to and away from its spindle pole, in 6-7μm excursions, with
velocities around 6µm/min, reversing directions at approximately 1-2 minute intervals as the bivalents as a whole either stretch or move off the equator [Fuge Cell Motil Cytoskel 13 (1989) 212]. To study force production during these oscillations we focused an ultraviolet microbeam (290nm wavelength) to a 2µm diameter circle and irradiated the spindle fibre between the kinetochore and pole. In some experiments, we irradiated single kinetochore fibres as the kinetochores moved to the spindle pole; in other experiments we irradiated single kinetochore fibres as the kinetochores moved away from the spindle pole. We expected that if we were to block kinetochore movement the kinetochore would stop moving at the position it was in at the time of irradiation. The irradiations indeed stopped the kinetochores from moving, but, much to our surprise, after the irradiation the kinetochores either continued movement to the pole at normal speeds until they reached the pole or, for those moving away from the pole at the time of irradiation, reversed direction and moved to the pole; only when they reached the pole did they stop moving. The kinetochore of its partner half-bivalent continued its normal oscillatory movement. The stopped kinetochores resumed oscillations after approximately 6 minutes, but the renewed excursions had decreased amplitudes. We plan to use immunofluorescence to determine which components of the irradiated spindle fibre are damaged by the irradiation.

1412/B570
Differential Targeting of Mitotic Cytoplasmic Dynein to Kinetochores and Spindle Poles.
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Cytoplasmic dynein is thought to function at kinetochores, spindle poles and the cell cortex; however, the basis of targeting to these distinct sites remains unclear. Supporting differential phosphorylation as one mechanism, we recently identified a novel phosphorylation site in the dynein intermediate chains (ICs), responsible for directing mitotic dynein to kinetochores (Whyte et al., 2008). In this study, we determined the impact of IC phosphorylation at a second site (Y130) and observed localization to spindle poles rather than kinetochores. PY130 dynein was observed at spindle poles from prophase to late anaphase and appeared different from dynein streaming poleward from kinetochores. To confirm this prediction, we assessed the impact of blocking dynein-driven streaming using nocodazole, taxol, and calyculin a which revealed no change in PY130 dynein at spindle poles. To define the requirements for spindle pole dynein localization, we disrupted candidate dynein binding proteins and assessed PY130 dynein. GFP-tagged NuMA colocalized extensively with PY130 dynein, and transfection of a NuMA phosphorylation site mutant resulted in ablation of PY130 dynein from spindle poles. Although this is consistent with a previous link between NuMA and dynein at spindle poles, PY130 dynein localized to spindle poles prior to the appearance of NuMA, suggesting that NuMA is not the primary dynein interactor. Dominant negative analysis of the dynein accessory proteins NudEL and CENP-F induced fragmented spindle poles and multipolar spindles; however, PY130 dynein localization to spindle poles was not affected. Motivated by a recently-discovered interaction between FAK and dynein, the impact of FAK inhibition was assessed, revealing disruption of spindle poles but normal levels of PY130 at pole fragments. These results suggest that some candidate dynein binding proteins are required for recruitment of dynein to spindle poles, whereas others are needed for dynein function in spindle pole integrity.

1413/B571
How Kinetochore-Microtubule Attachments Affect the Dynamic Behavior of Chromatin Bridges During Mitosis.
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Chromatin bridges represent one type of mitotic error that is believed to lead to chromosome rearrangements. Indeed, chromatin bridges are believed to break at random points during mitosis, thus generating “uncapped” chromosome ends that will tend to fuse with other uncapped ends, which will lead to what is known as Breakage/Fusion Bridge cycle. We hypothesize that chromatin bridges not always break during mitosis and that their mitotic behavior is dictated by a combination of chromosome mechanical properties and kinetochore-microtubule (kMT) dynamics. To test our hypothesis, we used stabilized human mammary epithelial cells (HMECs), which, due to their high degree of telomere dysfunction, frequently display chromatin bridges during mitosis. Using high-resolution microscopy, we found that the bridge KTs maintained the connection to kMTs in >90% of the cases. We also found that in 22.5% of the bridges one of the two bridge KTs exhibited a stretched appearance, and it was bound to a MT bundle that was significantly thinner than that bound to the KT at the other end of the bridge. In addition, we found that unstretched bridge KTs were more likely to exhibit EB1 positive staining than either non-bridge KTs or stretched bridge KTs. We next determined the position of bridge KTs in anaphase, telophase, and early G1 cells. In anaphase, the bridge KTs were positioned significantly closer to the equatorial region of the cell compared to non-bridge KTs. In telophase, the bridge KTs were “embedded” in the main nuclei in 50% of the cases, whereas in early G1 cells the bridge KTs could be seen separate from the main nuclei at one or both ends 75% of the time. Taken together, our data indicate that (i) the tension generated by the anaphase bridge is not sufficient to induce MT detachment; (ii) the stretching of some bridge KTs might depend on the inability of the respective k-fiber to polymerize in response to the pulling forces exerted by the chromatin bridge during anaphase B spindle elongation; (iii) frequently the chromatin connecting the two ends of a bridge does not break, and the persistent chromatin bridge pulls the two ends inward strongly enough to move the KTs at the end of the bridge away from the main nuclei.

1414/B572
Evaluating the Contribution of Lagging Chromatids to Chromosome Mis-segregation Associated with Chromosomal Instability (CIN).
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Most solid tumors are aneuploid and mis-segregate chromosomes at high rates in a phenomenon called chromosomal instability (CIN). CIN cells display high levels of lagging chromosomes due to merotelic attachment of microtubules to kinetochores, and this is a major mechanism for chromosome mis-segregation in these cells. However, not all lagging chromosomes segregate to the wrong daughter cell and we sought to determine the contribution of lagging chromosomes to the overall chromosome mis-segregation rate in human cells. To track chromosome segregation, we developed chromosomally stable HCT116 cell lines with a single chromosomally marked locus using a lacIGFP/lacO system. We then determined the mis-segregation and lagging chromatid rates following the induction of high levels of merotely using either drug washout strategies or depletion of mitotic kinesins. Nocodazole or monastrol washouts and depletion of Kif18a, MCAK, Kif2b, and CENPE all increase the rates of lagging chromosomes and chromosome mis-segregation. Interestingly, under all conditions, when the marked chromosome segregates to the wrong daughter cell, it most often resides inside the main nucleus and not as a micronucleus which is the common fate of a lagging chromosome during anaphase. When the marked chromosome did reside in a micronucleus, it was more likely to be in the correct daughter cell rather than the wrong daughter cell with its sister. These data have several implications. First, mis-segregation rates following drug washouts are significantly higher than in cells depleted of proteins involved in error correction, indicating the error correction machinery is easily overwhelmed by high levels of merotely formed by disrupted spindle geometries. Next, chromosomes segregate to the wrong daughter cell through mechanisms that may not involve an overtly obvious lagging chromosome. Thus, whereas lagging chromosomes may not frequently mis-segregate, high levels of lagging chromosomes are indicative of cellular state where kinetochore-microtubule attachments are excessively stable and predispose cells to high chromosome mis-segregation rates.
1415/B573

**A Mechanistic Link Between Whole-chromosome Aneuploidy and DNA Damage.**

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Cancer is associated with aneuploidy--both structural defects in chromosomes and abnormal numbers of intact chromosomes. The contribution of chromosome breaks to tumorigenesis is well accepted because of the possibility of generating cancer-causing mutations. However, the role of alterations of intact chromosome numbers remains a subject of debate, principally because mechanisms by which whole chromosome aneuploidy would affect cancer pathogenesis are poorly understood. We now have evidence to support one mechanism by which whole chromosome aneuploidy can lead to DNA breaks, via mis-segregation of chromosomes into micronuclei, structures commonly observed in cancer cells. Micronuclei containing intact chromosomes can form after mitotic errors, when the nuclear envelope reforms around a lagging chromosome. We have developed procedures to monitor the fate of micronuclei. Strikingly, whole chromosome-containing micronuclei develop evidence of DNA breaks during the first S phase after they are formed. Blocking DNA replication blocks the formation of these breaks. Defective DNA replication may be due to defective nucleocytoplasmic transport because micronuclei display a marked defect in assembling nuclear pore complexes. These findings suggest one mechanism by which mitotic errors result in DNA damage and thus potentially promote tumorigenesis.

1416/B574

**Mechanisms of Chromosome Instability in Cancer Cells.**

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Most cancer cells mis-segregate chromosomes at high rates during cell division, a phenotype commonly referred to as Chromosome Instability (CIN). We used colorectal cancer cells to identify chromosome segregation defects potentially responsible for CIN. Using a combination of high-resolution confocal microscopy and live-cell imaging, we found that CIN cells frequently assemble multipolar spindles in early mitosis, but they bi-polarize before anaphase onset and segregate their chromosomes in a bipolar fashion. Moreover, bipolar anaphase CIN cells exhibited high rates of merotely attached lagging chromosomes (i.e., chromosomes that lag behind during anaphase, and whose kinetochore is bound to microtubules from both spindle poles rather than just one). Finally, we found that multipolar prometaphase cells possessed higher numbers of merotely oriented kinetochores compared to bipolar prometaphases. Our data suggest a model by which merotelic kinetochore attachments can easily be established in multipolar prometaphases. Most of these multipolar prometaphase cells would then bi-polarize before anaphase onset, and the residual merotelic attachments would produce chromosome mis-segregation due to anaphase lagging chromosomes. We next asked if this phenomenon is common to other aneuploid cancer cells, and selected additional human cancer cell lines, including H460 (lung), MCF-7 (breast), and HeLa cells (cervix), in which we determined the frequency of multipolar spindles in prometaphase and anaphase cells, as well as the frequency of lagging chromosomes in bipolar anaphases. Our results showed that all cell lines analyzed exhibited similar frequencies (8-11%) of multipolar prometaphases, but lower frequencies of multipolar anaphases. We also found high frequencies of anaphase lagging chromosomes in all cell lines. Our data clearly show some common features among cancer cells derived from different sites and suggest that transient spindle multipolarity followed by spindle bi-polarization might promote kinetochore mis-attachment and chromosome mis-segregation later in mitosis in cancer cells of different origin and at different stages of transformation.

1417/B575

**Mitosis in the Minimal Eukaryote Ostreococcus tauri.**

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Eukaryotic chromosome segregation is coordinated by mitotic spindles, which range from simple intranuclear spindles in yeast to the complex cell-sized spindles of metazoans. All of the spindles observed so far share a canonical ultrastructure that has at least one kinetochore microtubule per chromosome. The most basic chromosome segregation machine probably operates in the smallest and simplest eukaryote, the picoplankton *Ostreococcus tauri*, which has just one mitochondrion, one chloroplast, and one nucleus at the start of the cell cycle. Here, we used electron tomography to image artificially synchronized mitotic *O. tauri* cells. No canonical mitotic spindles could be found in any of the approximately two hundred cells imaged. Furthermore, the chromosomes do not condense into discrete chromatids and appear to finish segregating by 'nucleokinesis' - a pinching off of the nucleus that is reminiscent of cytokinesis.

1418/B576

**The Role of Nups in Mitosis: A Systematic RNAi Screen in Drosophila S2 Cells.**

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The nuclear pore complex (NPC) is a large proteinaceous structure composed of multiple copies of 30 proteins termed nucleoporins (Nups). During interphase, the NPC's main role is the bidirectional transport of soluble molecules between the nucleus and cytoplasm. In higher eukaryotes, entry into mitosis is coincident with nuclear envelope breakdown, NPC disassembly and dispersion of Nups. Recent studies have demonstrated that some Nups have functions in mitosis. In order to probe the function of all nucleoporins in mitosis we carried out a comprehensive RNAi screen in S2 cells. We performed automated live and fixed analysis using S2 cells expressing mCherry-tubulin and H2B-GFP. Of the 29 dsRNAs used, 5 showed mitotic index alterations, spindle morphology and/or chromosome alignment defects. Further live cell imaging of the positive hits was carried out using S2 cells expressing a variety of markers. We have focused our subsequent studies on the nucleoporin ALADIN. Mutations of the ALADIN gene were initially identified as a cause of AAA syndrome, before it was recognized that the protein is part of the nuclear pore complex. We find that ALADIN-depleted cells display chromosome alignment and segregation defects and shortened mitotic spindles. In Aladin depleted cells the rate of microtubule flux in the mitotic spindle is significantly increased over control cells, possibly explaining the short spindle phenotype. Depleting the microtubule depolymerizer Klp10A along with ALADIN returns the rate of microtubule flux and spindle length to wild-type levels. GFP-ALADIN targets to an area around the mitotic spindle, but we do not see any direct localization at the centrosome or spindle pole. Further experiments are underway to determine the role that this nucleoporin plays is spindle formation, and whether the expression of ALADIN containing the disease mutations will also affect the mitotic spindle.

1419/B577

**Molecular Analyses of TIP60 Function in Cell Division Control.**

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The promyelocytic leukemia (PML) protein orchestrates the PML nuclear body dynamics and plasticity. Our recent studies show that TIP60 interacts with PML3 in nuclear body and plays an important role in UV-irradiated DNA damage response (Oncogene 2008. 27: 931; Journal of Biological Chemistry 2009. 284: 8747). To examine the potential role of TIP60 in cell division and chromosome plasticity, we carried out siRNA-mediated repression of TIP60 protein expression. In those TIP60-suppressed cells, we observed defects that were consistent with aberrant activation of the spindle checkpoint, such as premature sister-chromatid separation, chromosome bridges and mis-segregation in anaphase. In addition, suppression of TIP60 compromised the spindle...
assembly checkpoint as cells lacking TIP60 failed to arrest in mitosis when exposed to microtubule inhibitors, yielding interphase cells with multinuclei. Currently, we are carrying out proteomic analyses of proteins selectively repressed in response to TIP60 deficiency and delineating molecular pathways underlying TIP60-mediated checkpoint control in mitosis.

1420/B578
A Novel Role for the MRE11-RAD50-NBS1 (MRN) Complex in Mitosis.
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Faithful duplication of the genome in S phase followed by its accurate segregation in mitosis is key to maintain genomic integrity. The evolutionary conserved MRE11-RAD50-NBS1 (MRN) complex is involved in DNA damage repair and signaling, telomere stability and cell cycle control. Recently a cross-talk between the DNA damage pathway (BRCA1, BARD1, ATM) and the mitotic spindle assembly checkpoint have been suggested. The aim of our study is to evaluate the role of MRN complex in mitosis. Using *Xenopus Laevis* egg extracts we show that immunodepletion of the MRN complex from or MRE11 antibody addition to the extracts result in metaphase chromosome alignment defects. Spindle defects are also observed in mitotic-arrested egg extracts treated with the MRE11-specific inhibitor Mirin. In mammalian cells, to bypass the lethality associated with the disruption of any component of the MRN complex, we used the inhibitor Mirin to follow the cell progression in mitosis. We show that in contrary to mock treated cells that exhibit normal mitotic progression, a substantial number of cells treated with MRE11 inhibitor are blocked in metaphase and failed to start anaphase within 2h. We will discuss the role of the MRN complex in spindle formation, kinetochore-microtubule capture and microtubule dynamics.

1421/B579
Identification of a Novel Cell Cycle Gene by a Targeted RNAi Screen.
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To identify novel cell cycle genes, we have utilized a global meta-analysis of DNA microarray experiments. This approach is based on the probable association of co-regulated genes. Thus, groups of genes that share a common biological function tend to be co-expressed together. By searching the database for unknown genes that are co-expressed at the same time with known cell cycle regulators, we have identified candidate genes that likely share similar functions. To test the candidate genes, we have designed short hairpin RNA expressing vectors to target each gene for silencing. These expression vectors were then transiently transfected into HeLa cells stably expressing histone H2B fused to GFP, and live-cell imaging was used to detect gross changes in mitotic phenotypes compared to controls. One of the positive results from the screen identified a novel cell cycle gene. Knockdown of the gene product caused mitotic arrest of cells, followed by scattering of chromosomes. We further investigated the gene by creating a GFP fusion to determine the localization of the protein. In interphase cells, the protein localized to the nucleus with highly concentrated levels found in nucleoli. During mitosis, the protein concentrated at the perichromosomal layer and remained there until telophase. We hypothesize that the product of this gene represents a novel component of cell cycle regulation in mitosis originating from the perichromosomal layer.

1422/B580
A Model for Mitosis Progression in Budding Yeast.
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Chromosomes must establish bipolar attachments during mitosis before the cell can proceed safely to anaphase, but little is known about the timeline of events between chromosome replication and the moment all sister chromatids have finally established bipolar attachments. In this work, we have undertaken a quantitative study of mitosis progression in budding yeast by a combination of genetics, microscopy images and computer-vision methods for image analysis. Our results offer a completely new view of the spindle structure progression during budding yeast mitosis. After S phase, chromosomes are quickly captured by one of the spindle pole bodies and have a large percentage of syntelic attachments. Next, there is a period in which the spindle pole bodies begin to separate, but kinetochores feel no tension and their attachments experience a continuous turnover, are symmetrized and approximately 50% remain syntelic. Once the spindle pole bodies are sufficiently apart (~1 µm), bipolar attachments can generate enough tension and are stabilized. At that moment, approximately 50% of the attachments are bipolar. From then on, the remaining syntelic attachments are gradually and slowly resolved until all of them are bipolar and anaphase onset is triggered. Budding yeast cells spend thus most of the time during mitosis in prometaphase, establishing bipolarity, and have a very short metaphase. We also show that, besides cross-linking of microtubules, one of the roles of the kinesin cin8 is to regulate the kinetochore microtubule length. As a result, kinetochores in budding yeast cells adopt the characteristic bilobed distribution, even though it arises from a combination of kinetochores with syntelic and bipolar attachments. In view of our new results for the progression of the spindle structure the role of many kinetochore components will need to be reinterpreted.

Cytokinesis II (1423 – 1440)

1423/B581
Anillin is Required for Actomyosin Organization During Polar Body Extrusion in C. elegans.
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Cytokinesis is the essential process by which one cell physically divides into two. Due to its importance, redundant mechanisms ensure its success but confound efforts to define its basal requirements. Polar body extrusion (PBE) is the cytokinetic event that terminates maternal meiosis. Perhaps due to its asymmetric geometry, wherein most cytoplasm is retained by one daughter cell (the oocyte), PBE exhibits fewer redundant mechanisms and displays sensitivities that allow insight into the general mechanisms of cytokinesis. Specifically, Anillin, which has poorly-understood roles in cytokinesis across phylogeny and is dispensable for mitotic cytokinesis in C. elegans, is essential for PBE in this same system. We are using the C. elegans early embryo to understand Anillin and PBE. In C. elegans, unlike vertebrates, meiotic spindle anchoring is actin-independent, allowing perturbation and study of the actomyosin cytoskeleton during PBE. Fixed and live imaging revealed that myosin is asymmetrically distributed around the meiotic contractile ring as in mitosis, but when Anillin is depleted, myosin distribution is more even. Comparison with septin depletion suggests that ring asymmetry is not essential for PBE. Live imaging of GFP-tagged myosin revealed that during closure, the initially planar contractile ring forms a tall, persistent collar-like midbody. Following Anillin depletion, the ring stays flat as it closes. Anillin may aid PBE by formation of the tall midbody collar, via its role as a cytoskeletal crosslinker, or simply as an F-actin bundler. Imaging fixed embryos stained for F-actin, tubulin and DNA, we found that the collar-like midbodies in control cells comprise a stack of F-actin containing rings. Anillin-depleted embryos only rarely displayed midbody rings, and polar bodies were always abnormal: large, with wide necks, or failed completely. Constriction kinetics are not affected by Anillin depletion, indicating that depleted embryos fail PBE at a late stage. Our results suggest that Anillin-dependent actomyosin organization in the meiotic contractile ring ensures a large, stable contact with midbody microtubules, and thus successful PBE.
**1424/B582**

**Structural Memory in the Contractile Ring Makes the Duration of Cytokinesis Independent of Cell Size.**

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Cytokinesis is accomplished by constriction of a cortical contractile ring. We explore the inherent properties of the contractile ring by investigating how the rate of ring closure scales with respect to initial ring size. We show that for the first five embryonic divisions in *C. elegans*, despite the wide range of initial perimeters, constriction completes in the same time. This is explained by a strict proportionality between the constant rate of constriction and initial size. The total amounts of Myosin II, anillin and the septins were found to decrease in proportion to ring perimeter. For Myosin II, this occurred in the absence of rapid exchange with the other pools. These indicate that the contractile ring progressively disassembles while retaining a structural memory that renders constriction time independent of its initial size. We propose that the contractile ring is built from units of fixed size, which shorten at a fixed rate.

**1425/B583**

**Probing the Role of Anillin in the Contractile Ring-to-midbody Ring Transition During Cytokinesis.**

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During cytokinesis, a dynamic actin- and myosin-based contractile ring (CR) assembles at the cell equator and drives ingestion of a cleavage furrow. The CR serves to reduce the cell diameter and to hand over the plasma membrane to a midbody ring (MR), a stable structure that connects the sister cells prior to the completion of cytokinesis. How the MR forms at the end of furrowing remains unclear. The scaffold protein Anillin is a prominent marker of both the CR and the MR and is a candidate regulator of the transition from CR to MR. Through its conserved domains, Anillin can interact with many furrow components, including myosin, actin and septins. We have begun to dissect the molecular mechanisms mediating the localization of Anillin to the CR and MR in Drosophila Schneider's S2 cells. Induced expression of GFP fusions of Anillin mutants lacking functional domains has revealed differences in the requirements for CR and MR localization. In particular, we describe a truncated Anillin mutant (Anillin-ΔN) that comprises the C-terminal Anillin-homology (AH) and pleckstrin homology (PH) domains, but lacks the N-terminal domains including those that bind myosin and actin. Anillin-ΔN localizes to the cleavage furrow at the appropriate time during anaphase, but does not rescue the phenotype induced upon RNAi-mediated depletion of endogenous Anillin: Myosin II fails to remain at the equator during furrowing resulting in unstable furrows that oscillate wildly from side to side and that regress without reaching the midbody stage. In cells induced to express Anillin-ΔN in the presence of endogenous Anillin, furrows remain stable during ingestion and MRs form normally. However, these MRs are devoid of Anillin-ΔN, which instead localizes to punctate and filamentous structures that are shed from the furrow region in the latter stages of furrow ingression. These punctate structures also contain Rho1 and the septin, Peanut, normal constituents of both the CR and MR. Our work shows that the C-terminal AH and PH domains are sufficient for CR localization, and highlights the importance of additional N-terminal regions for the normal behavior of Anillin at the CR-to-MR transition.

**1426/B584**

**Multiple Roles of the Anillin-Related Protein Mid1p in the Fission Yeast *S. pombe*.**

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The anillin-related protein Mid1p improves the fidelity of cytokinesis in *S. pombe* by helping to place the cytokinetic ring. Fission yeast cells are cylindrical in shape and normally assemble
cytokinetic contractile ring perpendicular to the long axis in the middle of the cell by condensation of nodes containing Mid1p located in a broad band around the equator. In cells lacking Mid1p, the contractile ring is often off center at odd angles to the long axis, because the interphase nodes are not located around the equator. When joined by myosin-II and actin, these misplaced nodes condense into long cables that often fail to make a complete ring. Wild type cellular morphology is also compromised in Δmid1 cells which are often bent, branched or show ectopic bulges. We investigated how Mid1p helps to position and organize nodes by defining its domain structure and studying the effects of deleting one or more domains. An expression screen revealed five soluble fragments of Mid1p, M1-1, M1-2, M1-3, M1-4 and M1-6, and an insoluble fragment M1-5 that account for whole length (920 residues) of the protein. Expression of Mid1p constructs containing single domain deletions, M1-1Δ, M1-2Δ, M1-5Δ or M1-6Δ, in Δmid1 cells corrected the defects to different extents. In general these constructs corrected septal placement/angular orientation and cellular morphology in parallel. M1-1Δ was exceptional, because the cylindrical morphology of the cells was close to normal, but the orientation and placement of the division plane were compromised similar to Δmid1 cells. Thus Mid1p has a role in maintaining cellular morphology independent, at least in part, of its role in positioning the cytokinetic division plane.

1427/B585
Tumor Suppressor 14-3-3 Regulates Cell Mechanics and Cytokinesis through Interactions with Microtubules and Rac.
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During cytokinesis, regulatory signals emanate from the mitotic spindle; however, what these signals are and how they lead to the spatiotemporal changes in the cortex structure, mechanics and regional contractility is not well defined in any system. To investigate pathways that link the microtubule network to the cortical mechanical changes that promote cytokinesis, we used a chemical genetic approach to identify genetic suppressors of nocodazole, a microtubule depolymerizer. We identified 14-3-3 and found that it is highly enriched in the cell cortex where it stabilizes microtubule tips, maintains normal cortical tension, modulates actin wave formation, and controls the symmetry and kinetics of cleavage furrow contractility during cytokinesis. Furthermore, 14-3-3 acts downstream of a Rac small GTPase (RacE), which controls the global cortex mechanics and cleavage furrow ingress dynamics by maintaining 14-3-3 and actin crosslinkers in the cortex. In summary, 14-3-3 is a cortical protein that acts downstream of microtubules and RacE to control cell mechanics and cytokinesis.

1428/B586
The influence of Environmental Exposure on Asymmetric Cytokinesis in the Rat Embryos.
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Objective: Lineage allocation in mammalian embryos involves asymmetric cytokinesis during compaction, a defining event in the generation of stem cells. The objective of these studies was to evaluate the impact of dioxins on the process of stem cell formation in 3.5 day rat embryos obtained from exposed dams. Methods: Embryos were isolated from 3 months old females that had received control (DMSO) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) treatment from E10 through the time of mating. Three dimensional confocal data sets were prepared from embryos labeled for chromatin (H33258), f-actin (rhodamine labeled phalloidin) and tubulin (mouse monoclonal anti alpha) to establish nuclear position and patterns of spindle orientation in 8-16 cell embryos. Results: Control embryos (n=9) exhibit an equal ratio of central (52%) and eccentric
(48%) nuclei with the majority of the latter (>80%) being anchored basally by f-actin. at mitosis, the f-actin anchor persists at an anastral basal pole whereas the distal spindle pole is astral and linked the apical/outer border of blastomeres. In contrast, embryos from dioxin exposed mothers (n=22) exhibit more eccentric nuclei at all blastomere borders (85%) and a decreased fraction are centrally disposed (15%). Mitotic figures from treated embryos display monastral spindles that are apically anchored and result in an impairment of asymmetric cytokinesis at compaction yielding cells of equivalent sizes. Conclusions: The results show that maternal environmental exposures impact tethering of nuclei by an actin dependent polarization that dictates spindle orientation conducive to the biogenesis of embryonic stem cells. Acknowledgments: Supported by NIH ES 15878, and The Scientific and Technological Research Council of Turkey-TUBITAK (G.A.).

1429/B587
**Metaphase Spindle Proteome Reveals Potential Furrow Formation Factors.**
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Cytokinesis is an important and fundamental process in the development of all organisms. The factors that establish the cleavage furrow have remained mysterious and have eluded many for over 130 years. In order to identify factors required for early steps in cytokinesis, mitotic spindles from synchronized Chinese Hamster Ovary (CHO) cells were isolated. Proteins enriched from isolated metaphase-enriched spindles were identified by multidimensional protein identification technology (MudPIT) in collaboration with the Yates Lab at Scripps. A comparative genomics analysis between the spindle and the midbody proteome (Skop et al., 2004), identified potential candidates. Preliminary results from multiple MudPIT data sets identified several hundred proteins with two or more peptide hits. We compiled the spindle proteome by averaging four MudPIT data sets. We prioritized our list of candidates by identifying proteins specific to the metaphase proteome, using Babelomics (http://Babelomics.bioinfo.cipf.es/) and Microsoft Access. We are particularly interested in membrane-cytoskeleton remodeling proteins, as these factors are likely involved in establishing and regulating the acto-myosin contractile ring. We are currently screening several homologs of the identified mammalian candidates in C. elegans using RNAi. Potential candidates include factors that function in furrow formation. We are further characterizing candidates using In Vivo microscopy, genetics and cell biological techniques.

1430/B588
**Anillin Plays an Integral Role in Defining the Equatorial Plane during Cytokinesis.**
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Cytokinesis is the process by which the cytoplasm is divided into two daughter cells during cell division. This process is driven by ingestion of the actin-myosin contractile ring, which is under the control of RhoA. The plane of division is dictated by the position of the mitotic spindle by two pathways: A stimulatory pathway derived from central spindle microtubules, and an inhibitory pathway derived from astral microtubules. How these pathways determine the equatorial plane is not fully understood. Disrupting the central spindle results in the broad cortical localization of contractile ring components. However, these broad domains do not extend into the polar regions of the cell suggesting that another pathway restricts their localization. This pathway may be associated with astral microtubules since their disruption results in the cortical recruitment of contractile ring components, which are no longer restricted to a defined plane. We previously reported that central spindle disruption together with depletion of the actin and myosin scaffolding protein, Anillin, causes contractile components to spread over the entire cortex and into the polar regions of the cell. Thus, Anillin may communicate the position of the mitotic spindle and help establish the equatorial plane. Consistent with this hypothesis, Anillin transiently localizes to microtubules during early anaphase. This localization is dramatically enriched upon loss of active RhoA, as well as with microtubule stabilization. Therefore, RhoA activation coupled with microtubule dynamic instability switches Anillin from microtubules to the cortex where it is refined to a discrete cortical plane. Disrupting the central spindle by Aurora inhibition or MKLP1 depletion
in combination with microtubule stabilization restores the cortical localization of Anillin, but its localization varies from broad to discrete depending on the timing of the treatments. These results support that Anillin may signal between microtubules and the cortex and since Anillin is a scaffold for actin and myosin, can establish the equatorial plane for contractile ring formation.

1431/B589
Identification of Novel Regulators of Cytokinesis.
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Cytokinesis describes the division of cytoplasm to produce two daughter cells, due to the formation and ingression of an actin-myosin contractile ring. The position and assembly of the contractile ring is directed by the central spindle during anaphase. The RhoGEF Ect2 localizes to the central spindle in a Cyk-4 (MgcRacGAP)-dependent manner where it activates the GTPase RhoA, resulting in the formation of the contractile ring in the central plane of the cell. Anillin is a conserved protein that contains N-terminal actin and myosin binding domains as well as a C-terminal AHD (Anillin Homology Domain) region that binds to RhoA, Ect2 and Cyk-4 and a PH (Pleckstrin Homology) domain that interacts with Septins and phospholipids. Anillin plays a crucial role in the localization of active RhoA and is an early marker for the location of contractile ring in many different eukaryotic cells. This evidence, coupled with Anillin’s interactions with contractile ring components, the central spindle and the overlying membrane, support the hypothesis that Anillin is a global regulator of cytokinesis. To further understand the pathways that regulate cytokinesis, a mass-spectrometry approach was used to identify potential interactors of human Anillin. Using bacterially expressed AHD and C-terminal fragments of Anillin, pull down assays were performed with anaphase-synchronized cell lysates. Preliminary results indicate that the co-purified proteins are GTPase regulators, cytoskeletal, mitotic, signaling and vesicle proteins. These many different types of proteins support our hypothesis that Anillin is a global regulator of cytokinesis and we will further characterize some of these proteins for their roles in cytokinesis.

1432/B590
Anillin Stabilizes Active RhoA during Cytokinesis.
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Anillin is a conserved actin and myosin binding protein that marks the location of the division plane in many eukaryotic cells. In cultured human cells, Anillin co-localizes with RhoA and contains a highly conserved and essential region in the C-terminus (AHD or Anillin Homology Domain) that directly interacts with RhoA. When Anillin is depleted, the pool of active RhoA as visualized by trichloroacetic acid (TCA) fixation, is lost from the furrow. Anillin depletion causes a unique phenotype, where the contractile ring partially ingresses followed by repeated oscillations of the cytosol from side to side. This phenotype likely arises due to the uncoupling of active myosin from the equatorial plane of the cell. The inability to maintain myosin in the centre of the cell, coupled with a decrease in TCA-stabilized RhoA supports a feed back role for Anillin in the production of active RhoA at the furrow. Further characterization of Anillin-depleted cells revealed a second phenotype, where the contractile ring failed to form and ingress. This phenotype is reminiscent of Ect2 (RhoGEF) depletion, suggesting that the levels of active RhoA are reduced in these cells. Consistent with this hypothesis, we observed an interaction between Anillin and Ect2 In Vitro and in vivo. We mapped minimal binding domains on both Anillin and Ect2, and identified a key residue in Ect2 that mediates their interaction. Furthermore, we found that this interaction likely is required for Ect2 function in vivo. We propose a model where the central spindle localizes and activates Ect2, which initiates the recruitment of Anillin and contractile ring components to the equatorial plane. Anillin crosslinks components of the contractile ring, including actin and myosin, in addition to reinforcing the production of active RhoA in the central plane of the cell by interacting with both RhoA and its upstream regulator Ect2.
1433/B591
Inhibition of Cortical Myosin Recruitment by Astral Microtubules.
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During cytokinesis, a single cell separates into two genetically identical daughter cells by constriction of the actomyosin-based contractile ring. Regulation of the position and formation of the contractile ring are critical for successful cell division. The formation of contractile ring requires local activation of RhoA, which, in turn, promotes actin polymerization and myosin activation. The position of the contractile ring is determined by the position of spindle during anaphase. Upon anaphase onset, myosin is recruited to the cell cortex and coalesces to form large myosin foci that concentrate in the future plane of division where it drives membrane ingression. Two mechanistically distinct pathways redundantly regulate cortical myosin recruitment during cleavage furrow formation. In the first pathway, central bundles positively promote local recruitment of myosin; whereas in the second pathway, astral microtubules locally inhibit accumulation of cortical myosin. Previous studies indicate astral microtubules inhibit myosin accumulation in their immediate vicinity. However, the molecular basis of this local inhibition is not yet known. Here, we demonstrate that the cytokinetic scaffold protein anillin (ANI-1) regulates local myosin recruitment during anaphase. By using high-resolution imaging, we find that the behavior of cortical myosin in the astral microtubules-abundant area is similar to the behavior of the entire cortex in anillin-depleted embryos. Specifically, coalescence of cortical myosin is blocked and myosin remains in tiny, discrete foci. Therefore, astral microtubules may locally inhibit anillin function. Furthermore, imaging of both live and fixed C. elegans embryos indicate that anillin associates with astral microtubules. In addition, we show that anillin mediates mechanical interactions between microtubules and membrane associated proteins. These data indicate that microtubules sequester anillin from the cortex, thereby locally inhibiting cortical myosin recruitment.

1434/B592
Role of the Endoplasmic Reticulum in Division Site Placement in Cytokinesis.
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For proper cell division, the division plane must be appropriately positioned. Fission yeast Schizosaccharomyces pombe cells divide at a site determined by the position of the nucleus through a mechanism dependent on mid1p (an anillin-related protein). mid1p is a peripheral membrane protein and localizes to 70-100 cortical dots (midsomes) at a site overlying the nucleus; in mitosis, mid1p recruits Myosin II and other ring components to these cortical structures to guide the process of ring formation. We are interested in how mid1p is localized in this cortical pattern. Recent work has revealed that this pattern arises from a combination of multiple mechanisms: 1) nuclear shuttling, which may enrich mid1p in the vicinity of nucleus; 2) a polar gradient of the pom1p kinase, which inhibits mid1p from stably associating with the cortex at a non growing cell tip; 3) polar inhibition by growth at a growing cell tip, which inhibits mid1p via transport away from this tip; 4) other midsome components such as cdr2p, which stabilizes mid1p at the cortex. Here, we describe a surprising new player in this complex process: the endoplasmic reticulum (ER). Although it has been assumed that mid1p localizes to the plasma membrane, the cortical ER lies just beneath the plasma membrane. The ER is organized in tubule and sheet-like subdomains, and we find that mid1p dots localize to the junction of these ER subdomains a reticulon mutant that affects ER membrane organization contributes to defects in division site placement, demonstrating a function of the ER in this process. Thus, these studies highlight how the process of division site placement globally integrates the contributions of multiple processes throughout the cell.
The Nematode Worm Cytokinesis Formin CYK-1 is Regulated by Auto-Inhibition and GTP-RhoA Activation.
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Animal cells coordinate cleavage site positioning through both astral and spindle microtubules, but the mechanism of subsequent contractile ring assembly and constriction is poorly understood. In the C. elegans early embryo, the small GTPase RhoA is thought to promote contractile ring assembly by activating the actin nucleation factor formin CYK-1. Formins contain actin assembly formin homology 1 and 2 FH1FH2 domains, which are flanked on either side by N-terminal DID and C-terminal DAD regulatory domains. Some formins are regulated by auto-inhibition through association of their N- and C-terminal regulatory domains, and Rho GTPase activation. We utilized a combination of biochemical, single actin filament TIRF microscopy, genetic and cell biology assays to determine whether CYK-1 is auto-regulated through putative DID and DAD domains. We found that CYK-1 mediated nucleation, but not CYK-1 mediated actin filament elongation, is auto-inhibited through direct association of its N- and C-terminal regulatory domains. Point mutations of residues within the putative DID and DAD regulatory domains completely abolish auto-inhibition. Furthermore, CYK-1 can be partially activated by non-hydrolyzable GMP-PNP bound RhoA, as well as a constitutively active RhoA mutant. On the other hand, GDP RhoA, GMP-PNP and GDP Cdc42, or GMP-PNP and GDP Rac are unable to bind and activate CYK-1. Therefore, other mechanisms must be in place to fully activate CYK-1 and to turn off CYK-1 mediated actin filament elongation. We are now exploring the mechanism of auto-inhibition and activation in the context of full-length CYK-1.

Molecular Mechanisms of Formin Regulation during Cell Division.
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Formins assemble non-branched actin filaments and modulate microtubule dynamics during cell migration and cell division. At the end of mitosis, formins contribute to the generation of actin filaments that form the contractile ring. Rho small GTP-binding proteins activate mammalian Diaphanous-related (mDia) formins by directly binding and disrupting an intramolecular autoinhibitory mechanism. While the Rho-regulated activation mechanism is well-characterized, little is known about how formins are switched off. We have discovered a novel mechanism of formin regulation during cytokinesis based on the following observations: 1) mDia2 is targeted for disposal by post-translational ubiquitin modification at the end of mitosis; 2) mDia2 interacts with the anaphase-promoting complex/cyclosome activator Cdh1, which likely mediates mDia2 ubiquitination 3) both knockdown and constitutive activation of mDia2 yields multinucleate cells due to failed cytokinesis; and 4) the cytokinesis block is dependent upon mDia2-mediated actin assembly, because versions of mDia2 incapable of nucleating actin, but that still stabilize microtubules, have no effect on cytokinesis. We propose that the tight control of mDia2 expression and activity is essential for the completion of cell division.

A Pathway Screen: The Discovery of Small Molecule Inhibitors of the Rho Pathway Using a Combination of Chemical Genetics and RNAi.
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The Rho pathway is a central regulator of diverse cellular processes involving the actin cytoskeleton, including contraction, migration, adhesion and cytokinesis, the last step in cell division. Rho pathway proteins have been implicated in different types of cancer. Currently, very
few small molecules that specifically target the Rho pathway exist. We report a study where we developed a method to target a small molecule screen towards a specific pathway. We conducted a phenotypic screen of approximately 38,000 compounds from commercial sources and natural product extracts to identify small molecules that target the Rho pathway, focusing on the role of Rho in cytokinesis. In a chemical genetic variant of a classical modifier screen, we used RNAi to sensitize cells and identified small molecules that suppress or enhance the RNAi phenotype. We have discovered promising candidate molecules that interact differently with proteins in the Rho pathway. We show that selected compounds inhibit a function of the Rho pathway in cells: the correct localization of phosphorylated myosin to the cleavage furrow during cytokinesis. Small molecules from our screen will be important tools to dissect in more detail how Rho signaling regulates dynamic reorganizations of the actin cytoskeleton. Our screening approach should be widely applicable to study any signaling pathway.

1438/B596
The Nucleoporin Nup153 Has Separable Roles in Both Early Mitotic Progression and the Resolution of Mitosis.
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Cells divide and accurately inherit genomic content through coordinated changes in cellular organization and chromosome dynamics. Recent studies have found examples of nucleoporins that contribute to the coordination of nuclear envelope remodeling, chromosome segregation, and mitotic progression in a manner distinct from their canonical roles in nucleocytoplasmic trafficking and nuclear pore architecture. Thus, nucleoporins are emerging as an important resource for coordinating mitotic events. We have found that the human nucleoporin Nup153 functions at two stages during mitosis. Reduction of endogenous Nup153 levels in HeLa cells results in a delay during the late stages of mitosis accompanied by a severe persistence of unresolved cytokinetic midbodies. Depletion of Nup153 to an even lower threshold leads to an increase in multilobed nuclei and a pronounced defect earlier in mitosis. To address whether the phenotypes observed are different manifestations of the same defect or whether they represent separate functions for Nup153, we used a rescue strategy. This revealed that distinct Nup153 constructs complement the early and late mitotic defects, thus demonstrating that two mitotic functions for Nup153 can be uncoupled. Rescue of the midbody phenotype requires the FG-repeat domain, a region known to contribute to the transport function of the nuclear pore complex. At these levels of Nup153 depletion, however, protein trafficking is not significantly affected, suggesting a specialized role for the Nup153 FG-repeat domain during late mitosis and/or mitotic exit. Together, our results indicate that the nucleoporin Nup153 is critical to the execution of mitosis and plays at least two separable roles at this time of the cell cycle.

1439/B597
Novel Role for the Transmembrane Protein GpIbα in Cytokinesis and Tetraploidization in Cancer Cells.
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GpIbα is a transmembrane subunit of the von Willebrand factor receptor on blood cell surfaces, functioning in platelet adhesion and activation. Recent researches have revealed that GpIbα also plays roles in transformation, genomic instability and senescence as an oncoprotein. Our immunofluorescence analysis demonstrated that GpIbα was colocalized with actin and filamin, an actin-binding protein, at the cleavage furrow in anaphase cells, suggesting a novel role for GpIbα in cytokinesis. Cytokinesis failure gives rise to tetraploid cells, an important intermediate in genetic instability and tumor formation. Interestingly, when we overexpressed GpIbα in non-cancer cells with p53 knockdown, GpIbα, filamin and actin became mislocalized. RhoA, an important cytokinesis regulator, was localized asymmetrically. Abnormal morphologies such as
blebbing were also observed, suggesting defects of cytokinesis from GpIbα overexpression. Indeed, live cell imaging revealed an elevated percentage of cytokinesis failure in GpIbα-overexpressing cells, in agreement with a higher percentage of binucleation shown by immunofluorescence. Taken together, our data indicated that GpIbα overexpression led to cytokinesis failure and tetraploidization. Conversely, in cancer cells which have been previously demonstrated to overexpress GpIbα, we observed decreases in percentages of binucleation and mitotic defects upon GpIbα knockdown, consistent with the hypothesis that GpIbα overexpression promoted genetic instability in cancer cells. In order to investigate the mechanism, the filamin-binding domain deleted GpIbα was overexpressed in non-cancer cells; but the percentages of cytokinesis failure and binucleation did not increase, indicating that binding to filamin was essential for GpIbα overexpression to inhibit cytokinesis. Collectively, our data suggest that the transmembrane protein GpIbα is involved in cytokinesis. Overexpression of GpIbα by tumor cells disrupts normal cytokinesis and results in tetraploidization and genetic instability.

**1440/B598**  
The Yeast Epsin and AP180 Families of Endocytic Adaptors are Involved in Cell Division and Septin Assembly.  
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We have recently reported that yeast epsin2 plays a role supporting cell division. This novel epsin function is mediated by Epsin N-Terminal Homology (ENTH) domain binding to the Cdc42 GTPase Activating Protein (GAP) and septin-organizing protein Bem3. Specifically, epsin2 was capable of downregulating Bem3 activity and contributing to its intracellular localization. Furthermore, overexpression of the ENTH domain of epsin2, led to severe defects in cell division and dramatic abnormalities in septin cytoskeleton organization. The ENTH2 determinants required for these effects were mapped to specific residues within ENTH domain loop7 and helix 8. Here we show that one member of the epsin-related AP180 family of endocytic adaptors (yAP1801) displays homology to ENTH2 loop7 within its AP180 N-Terminal Homology (ANTH) domain. Importantly, overexpression of the ANTH domain led to cell division abnormalities but with significantly less penetrance than ENTH2. Co-overexpression of ANTH/ENTH2 showed a synergistic effect upon the cell division abnormalities. These findings led us to hypothesize that yeast AP180 also participates in cell division pathways. In fact, experiments performed with different epsin- and AP180-deletion backgrounds revealed that ENTH2 effects on cell division require the presence of at least one member from the epsin or AP180 families of endocytic adaptors. The epsin and AP180 proteins are redundant for endocytosis, this work highlights another aspect of the functional redundancy between these protein families that involves cell division and septin assembly regulation in yeast.

**Kinetochores I (1441 – 1456)**

**1441/B599**  
De Novo Centromere Formation in C. elegans.  
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Chromosome segregation is a fundamental event pertinent to the origins of cancer and the development of cancer therapies. Centromeres are the specialized chromatin domains where kinetochores assemble to direct chromosome segregation. Although centromeric DNA sequences vary dramatically among eukaryotes, most kinetochore components are highly conserved. While previous studies have shown that a specific sequence is neither necessary or sufficient for centromere function, sequence characteristics can significantly bias neocentromere formation. All functional centromeres in monocentric or holocentric organisms, including neocentromeres, contain a histone H3 variant, CENP-A. CENP-A is thought to provide the epigenetic mark specifying centromere identity. However, how CENP-A is first recruited to the centromere, and how centromere is propagated through gametogenesis and mitotic divisions are not well
understood. Holocentric C. elegans provides a system to study de novo centromere formation. Naked DNA injected into the germline of C. elegans can be recombined and transmitted as extrachromosomal arrays through mitosis and meiosis at variable frequency. Cytological analyses of embryos show that these arrays recruit CENP-A, build kinetochore, exhibit a delay in congressing to the metaphase plate, but segregate along with endogenous chromosomes in anaphase. I am exploring factors that may affect array transmission stability. I am also utilizing different naked DNA substrates to examine the relationship between DNA sequences and centromere formation efficiency. This study will shed light on the requirement of centromere establishment and maintenance, and facilitate the development of artificial chromosomes.

1442/B600
Determination of the Structure and the Function of HsKNL2 MYB Domain.
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The centromere is the chromosomal locus on which the kinetochore assembles during cell division. Centromeric chromatin is composed of a histone H3 variant, CENtromere Protein a (CENP-A), which is essential for centromere specification and propagation. The assembly mechanism of CENP-A nucleosomes has been a topic of intense study, however the mechanism which specifies CENP-A to a particular chromosomal locus remains unknown. Recently, Kinetochore Null-2 (KNL-2) was identified in Caenorhabditis elegans (C. elegans) by functional genomics as required for CENP-A loading at centromeres. RNA interference (RNAi) of KNL-2 results in loss of CENP-A loading and localization to centromere, therefore failure in chromosome segregation during mitosis. Sequence analysis reveals only a MYB like DNA binding domain within KNL-2. In other well studied MYB proteins, this domain is known to bind DNA with high specificity. Here we show that the C. elegans KNL-2 MYB domain is able to bind genomic DNA in vitro. However, the function or significance of this activity is not known. We hypothesize that the KNL-2 MYB domain binds a specific DNA motif at centromeres and that is important for CENP-A loading to the centromere. To better understand the function of the MYB domain, we solved the 3 dimensional structure using Nuclear Magnetic Resonance (NMR). This revealed an expected helix-loop-helix-loop-helix structure. We are currently generating a co-structure in presence of DNA which will aid in determining specific residues important for DNA recognition and stabilization. We will generate point mutations to test In Vivo function by gene replacement in C. elegans and in HeLa cells and look for centromere localization of KNL-2 and CENP-A. The results generated in this project will help us to better understand the mechanism by which CENP-A nucleosomes are loaded to the centromere.

1443/B601
Aurora B is Required for Assembly of a Dynein-Binding Platform at Kinetochores.
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Kinetochores are required for several aspects of mitosis, including initial interactions between chromosomes and microtubules (MTs), chromosome movement and activities associated with the spindle assembly checkpoint (SAC). Cytoplasmic dynein plays a role in one or more of these kinetochore functions, however the specific contributions of dynein remain under investigation. The Vaughan laboratory recently identified a novel phosphorylation site in the dynein intermediate chains (ICs) that is required for initial recruitment to kinetochores during prometaphase (Whyte et al., 2008). This phosphorylation site undergoes dephosphorylation in response to MT attachment and kinetochore stretch at metaphase. Because PP1g is implicated in catalyzing this dephosphorylation, we investigated the role of Aurora B (AurB) as a potential kinase in the kinetochore dynein pathway. AurB inhibition was achieved using either the small molecule inhibitor ZM443749 or transfection of a dominant-negative kinase-dead AurB construct. The impact of AurB inhibition was assessed by immunofluorescence microscopy (IFM) analysis of
kinetochore proteins or live-cell imaging of mitotic progression. AurB inhibition reduced the recruitment of phospho-dynein to kinetochores substantially, raising the possibility that AurB phosphorylates dynein or a dynein-binding protein. This led to multiple types of premature anaphase onset. Western analysis revealed that AurB inhibition did not affect dynein phosphorylation directly, but rather affected the ability of kinetochores to recruit phospho-dynein. Assessing the chain of interactions needed for successful dynein recruitment, both zw10 and dynactin were reduced after AurB inhibition, suggesting that proteins that interface with dynein at kinetochores could be targets for AurB. In contrast, HEC1 was not affected suggesting that basic kinetochore structure is intact after AurB inhibition. These findings suggest that AurB activity is required for assembly of a dynein recruitment platform and that AurB could be involved in regulating the interactions between HEC1, zwint and zw10 during mitosis.

1444/B602
In Vitro Assays to Study Tracking of the Shortening Microtubule Ends and Measurement of Associated Forces.
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Accurate segregation of mitotic chromosomes relies in part on a strong linkage between the kinetochores and the plus ends of spindle microtubules (MTs). These attachments are maintained even as the MTs shorten from their kinetochore-associated ends, and despite the large variability in the magnitude of load from the chromosomal “cargo”. Analysis of the underlying mechanisms has been recently facilitated by the identification and purification of various kinetochore-complexes. To study the interaction of these protein complexes with the ends of shortening microtubules In Vitro we have developed a “segmented” MT technique, which allows quantitative characterization of the tracking of shortening MT ends by protein-coated beads, as well as controlled measurement of the associated forces. Surprisingly, there are quite a few protein complexes, including Dam1/DASH, Ndc80, kinesin-like proteins and Ska1-containing complexes that can couple MT depolymerization to bead motility. Studies with Dam1-coated beads demonstrate that at least two different mechanisms, one involving bead rotation and the other not, can in principle drive such motions. Since rolling beads are a poor model to study chromosome motility, additional tests should be carried out with each protein complex to reveal the mechanism by which it produces end-tracking bead motility. Here, we review the coupling properties of these protein complexes, highlighting their differences and similarities.

1445/B603
A Super-resolution Map of the Vertebrate Centromere.
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The centromere is a specialized region of the eukaryotic chromosome where in mitosis the kinetochore is assembled. This region, appears as a cytologically visible primary constriction on mitotic chromosomes of higher eukaryotes and it is fundamental for the equal segregation of sister chromatids into two identical daughter cells. Although the mechanism of segregation and kinetochore proteins are very well conserved between organisms, the characteristics of centromeric region are relatively divergent with one common theme: the presence of histone H3 variant (CENP-A) in the nucleosomes that define the centromere. Overall, the organization of this region is still poorly understood. In order to study chicken DT40 centromeres we analyzed condensin conditional KO cells (previously developed in the lab) in metaphase, where “naked” In Vivo centromeres can be visualized due to the striking reversible movements that kinetochores can undergo away from the body of the chromosomes when microtubules attach. To measure the amount of DNA present in the pulled CENP-A-containing chromatin we mixed in three
bacteriophages (Lambda, P1 and T4) with known amounts of DNA, fixed and stained with DAPI to calibrate and quantify. From our results we measured values ranging from 30 kb to 150 kb (with a mean value of 60 kb) in the CENP-A-containing chromatin. Furthermore, we also examined centromeres using an In Vitro assay where cells are placed in an EDTA containing buffer designed to be inefficient at neutralizing the excess negative charges on the DNA. In this buffer, chromatin higher-order structure unfolds to beads-on-a-string nucleosomes and with longer incubations CENP-A containing chromatin is also unfolded, whilst histone modifications are preserved. By tagging the CENP-A with the photo-switchable fluorescence protein Dronpa we were able to look at this region using super-resolution microscopy based on single-molecule switching, and simultaneously stain for different histone modifications or inner kinetochore proteins fluorescently labeled with Cy5. These results allow us to build a precise map of the vertebrate centromere and a potential model for centromeric chromatin folding.

**1446/B604**

**Loops, Rings, and Chromosome Segregation: Analysis of Centromere Structure in Candida albicans.**

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*Candida albicans*, a fungal pathogen of humans, has small, regional centromeres. Unlike *S. cerevisiae* point centromeres, kinetochore assembly is not dependent on DNA sequence, but rather is inherited epigenetically. However, compared to other characterized regional centromeres, *C. albicans* centromeres are smaller (2-3 kb) and lack pericentric heterochromatin. Our laboratory has recently demonstrated that neocentromeres can form in *C. albicans* following disruption of the native centromere (Ketel et al. 2009 *PLoS Genetics* 5(3):e1000400). Neocentromeres are adjacent to the native centromere in some cases and are on chromosome arms distal to the native centromere in other cases. The combination of the small centromere size and the formation of distal neocentromeres makes *C. albicans* an excellent model for studying the structure of regional centromeres. Previous work with *S. cerevisiae* has shown that a looped DNA structure is found near point centromeres. Using a chromosomal conformation capture (3C) assay, looped DNA is also found near *C. albicans* regional centromeres, suggesting that looped structures are a conserved feature of small point and regional centromeres. Cohesin proteins have important roles in modulating the structure of DNA and are enriched in large regions near *S. cerevisiae* centromeres. We examined the pattern of localization of the cohesin complex member Smc1p across *C. albicans* chromosomes using chromatin immunoprecipitation followed by microarray analysis (ChIP-chip). Smc1p is not enriched at the centromere central core DNA (which is bound by CENP-A), but is enriched in the surrounding region. This localization pattern is consistent with a model in which cohesins stabilize the centromere loop. To distinguish features of centromeres dependent on the native context of the centromere DNA from features influenced by functional kinetochore formation, we are comparing neocentromere DNA regions with and without functional centromeres. ChIP-chip of Smc1p in a distal noecentromere strain indicates that the localization pattern of cohesin is dependent on the presence of a functional centromere. We are currently testing the hypothesis that looped structures also are present at functional neocentromeres.

**1447/B605**

**Probing Homologous Chromosome Biorientation in the First Meiotic Division of Live Mouse Oocytes.**

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The faithful segregation of homologous chromosomes in meiosis I is essential for fertility and early embryonic development. A key step in this process is the biorientation of homologous chromosomes, which has to be established by the end of metaphase, when homologues are pulled to opposite poles by spindle microtubules attaching to their kinetochores. Despite the importance of this process, how structural changes and dynamic movement of chromosomes
lead to establishment of homologue biorientation is currently unknown. In this study, we therefore performed quantitative high-resolution 4D imaging of kinetochores marked with EGFP-CENP-C in live mouse oocytes. We recorded complete 3D reconstructions of all meiotic kinetochores at high spatial (0.25x0.25x1.5μm; xyz) and temporal (90sec) resolution over 9 hours covering the complete first meiotic division. This yielded very large data sets (5400 images/oocyte) that allowed us to track every kinetochore from shortly after NEBD until anaphase onset. This data provides first accurate 4D map of kinetochores in mouse oocytes and thus an unbiased resource to analyze kinetochore dynamics during meiosis I. Surprisingly, the kinetic profile and the absolute timing of homologous chromosome biorientation were very different among the different chromosomes in a single oocyte with deviations of up to two hours, suggesting that characteristics of individual chromosomes determine the efficiency of biorientation.

1448/B606
Sds22 Regulates Aurora B Activity and Microtubule-Kinetochoore Interactions at Mitosis.
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The kinetochore is a multi-protein machine that mediates the attachment of microtubules to chromosomes and the critical force generation and signalling systems necessary for proper chromosome segregation. Protein phosphorylation is used to regulate the function of kinetochore components. We have studied the function of Sds22, a conserved regulator of protein phosphatase 1 (PP1) activity, and determined its role in modulating kinetochore microtubule interactions and the protein kinase activity of Aurora B. Sds22 is required for proper progression through mitosis and localization of PP1 to mitotic kinetochores. Depletion of Sds22 causes an increase in phosphorylation on the T-loop of Aurora B and an increase in the rate of recovery from monastrol arrest. However, Sds22 depletion also causes a loss of phosphorylation on the Aurora B targets CENP-A, MCAK, and Hec1, suggesting a loss of Aurora B activity. In Sds22 depleted cells, there is a marked change in the binding affinity of Aurora B for the inner centromere, suggesting that a critical determinant of substrate phosphorylation at the kinetochore is the dynamic turnover of Aurora B at the centromere. This regulatory pathway affects kinetochore mechanics: depletion or over-expression of Sds22 resulted in a respective increase or decrease in sister kinetochore distance, consistent with the changes observed in phosphorylation of kinetochore components and motors. Using a FRET-based assay to probe the interaction between the kinetochore and the microtubule, we demonstrated that Sds22 depletion perturbs the interaction between Hec1 and the microtubule lattice. This perturbation caused an activation of the spindle assembly checkpoint, which explains the mitotic delay observed in Sds22-depleted cells. These results demonstrate that Sds22 functions as a critical regulator of targeting of PP1 to the kinetochore, Aurora B to the centromere, and force generation at the kinetochore-microtubule interface.

1449/B607
Roles for Casein Kinase 2 and Aurora B in Kinetochore Regulation.
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The kinetochore is a multi-protein complex that assembles on the centromere and attaches chromosomes to spindle microtubules during cell division. Kinetochore attachments are tightly monitored to ensure faithful chromosome segregation. A recent genome-wide yeast two-hybrid screen focusing on the mitotic spindle identified interactions between the essential kinetochore protein Mif2 (S. cerevisiae homolog of human CENP-C) and two Casein Kinase 2 (CK2) subunits, Cka2 and Ckb2. CK2, a ubiquitous and highly conserved kinase in eukaryotes, is composed of two catalytic subunits (Cka1 and Cka2) and two regulatory subunits (Ckb1 and Ckb2). Although CK2 has been linked to a variety of cellular processes, how CK2 regulates these diverse events...
is largely unclear. In this study, we investigated the roles of CK2 in mitotic spindle function. Consistent with yeast two-hybrid results, mass spectrometry analysis revealed that CK2 co-purified with Mif2. Furthermore, we showed that Mif2 and another essential kinetochore protein, Cbf2 (also called Ndc10), are phosphorylated by CK2 In Vitro and in vivo. Cbf2 is a component of the CBF3 complex that directly binds to centromere DNA and initiates kinetochore assembly, whereas Mif2 serves as an important link between the inner and central kinetochore. These results suggest that CK2 plays a direct role in regulation of the kinetochore. Indeed, loss of Cka2 kinase activity prevents anaphase spindle elongation and proper chromosome segregation. In addition to phosphorylation by CK2, Mif2 and Cbf2 are known to be phosphorylated by the yeast Aurora B kinase. We found that Mif2 phosphorylation by CK2 plays an antagonizing role to its phosphorylation by Aurora B, whereas Cbf2 phosphorylation by CK2 enhances the effect of its phosphorylation by Aurora B. Our results demonstrate that CK2 regulates mitosis and provide evidence for distinct regulation of two kinetochore proteins by different kinases.

1450/B608
The Dam1 Complex is a Processivity Factor for the Ndc80 Complex on Dynamic Microtubules.

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During mitosis, kinetochores form and maintain load-bearing attachments to the plus ends of dynamic microtubules. How this attachment grips the end of a microtubule as thousands of tubulin dimers are added or removed remains unclear. Genetic, biochemical, biophysical and structural studies implicate both the Ndc80 and Dam1 complexes as essential points of direct contact between kinetochores and microtubules in budding yeast. Using techniques for manipulating and tracking individual molecules in vitro, we demonstrated that each complex acting alone can form dynamic, load-bearing attachments to assembling and disassembling tips. Ndc80 complex-based coupling likely occurs through a biased diffusion mechanism and this activity is conserved from yeast to humans. The Dam1 complex is required for kinetochore coupling to microtubule tips in vivo. Earlier genetic studies showed that Dam1 complex association with kinetochores is dependent on the Ndc80 complex, and that the two complexes interact. However, we detect only a weak interaction between the Dam1 and Ndc80 complexes in solution as assayed by velocity sedimentation. Using total internal reflection fluorescence microscopy and recombinant GFP-tagged Ndc80-complex we establish a microtubule-dependent link between the Ndc80 and Dam1 complexes. Through this interaction, the Dam1 complex enhances the ability of the Ndc80 complex to track with disassembling microtubule tips. We further show that this interaction is directly regulated by the Aurora kinase Ipl1, a key signaling component of the spindle assembly checkpoint. We therefore propose that the Dam1 complex is a regulated processivity factor for the Ndc80 complex in vivo.

1451/B609
Regulation of hZw10 Localization to Tensionless Kinetochores by Aurora B Kinase Activity and hZwint-1 Interaction.

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In order to maintain faithful chromosome segregation, the mitotic checkpoint monitors kinetochore-microtubule attachments as well chromosome alignment through tension across sister kinetochores by accumulating checkpoint proteins at kinetochores. We have shown that the checkpoint protein hZw10 accumulates at tensionless kinetochores following low-dose taxol treatment. This accumulation of hZw10 is consistent with the observed reduction in hZw10 dynamics at metaphase kinetochores following low-dose taxol treatment. We also found that inhibition of Aurora B kinase activity with ZM447439 prevented hZw10 accumulation (Famulski (2007) CurrBiol). hZw10 contains 3 consensus Aurora B phosphorylation sites (Serine 25, Serine...
103 and Serine 322). We have mutated each site individually as well as concurrently to produce mutants that either mimic phosphorylation (aspartic/glutamic acid) or mimic the unphosphorylated state (alanine). We are examining the mutants for their ability to localize to kinetochores, to kinetochores that lack MT attachments as well as at tensionless kinetochores. Those mutants that are able to accumulate at the tensionless kinetochores will be examined further to determine if they are responsive to Aurora B kinase activity inhibition. In addition to being regulated by Aurora B kinase activity, hZw10 checkpoint activity is regulated by its interaction with the structural kinetochore protein hZwint-1 (Famulski (2008) JCB). We will determine if interaction with hZwint-1 is required for tension sensitive accumulation of hZw10 at kinetochores by examining hZwint-1 non-interacting mutants of hZw10.

1452/B610
Dissecting the Human Kinetochore - Chromatin Interface.
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Accurate cell division requires the interaction of chromosomes with the mitotic spindle via the centromere, where the macro-molecular kinetochore complex forms. To avoid mis-segregation, it is crucial that the kinetochore forms only once on each chromatid. As the formation of the kinetochore is restricted to the centromere, a long-standing question has been the nature of this site, and the genetic and molecular factors that define the region of kinetochore assembly. The mammalian centromere is loosely defined by arrays of repetitive alpha-satellite DNA sequences. However, these sequences alone do not guarantee kinetochore formation, and additional epigenetic factors likely play a role in defining the region. Several proteins are known to localize constitutively to the centromere in human cells (collectively known as the ‘constitutive centromere network’ - CCAN), and are required for correct assembly of the outer kinetochore. To better understand the processes of centromere specification and kinetochore assembly, we are using a combination of biochemical and cell biological approaches to dissect the interactions of the CCAN proteins with the chromatin and the outer kinetochore. We have reconstituted several CCAN sub-complexes in vitro, and are currently defining the interactions of these proteins within the CCAN network, and with centromeric DNA. By combining this biochemical data with parallel In Vivo studies, we aim to further elucidate the structure of the kinetochore - chromatin interface, and the mechanisms behind centromere specification.

1453/B611
Cyclin-Dependent Kinase is Controlling the Timing of CENP-A Assembly.
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Centromeres are specialized chromosomal domains required for proper kinetochore assembly and consequently for accurate chromosome segregation during mitosis. Centromere protein a (CENP-A), a histone H3 variant present exclusively at centromeres, is considered an epigenetic mark responsible for centromere identification and function. Unlike canonical histones, that are assembled during DNA replication throughout chromatin, the assembly of nascent CENP-A into centromeric nucleosomes is restricted to early G1 phase of the cell cycle. Entry into G1 is dictated by loss of Cyclin-Dependent Kinase (CDK1) activity through APC dependent degradation of its activator cyclin B. Using small molecule inhibitors in combination with a unique method to visualize nascent CENP-A (SNAP-tagging) we have now found that this major regulator of mitosis is also controlling the timing of CENP-A assembly. Premature inhibition of CDKs in G2 cells leads to unscheduled CENP-A assembly without passage through mitosis and without the need for APC activation. Consistent with this we see rapid recruitment of hMis18α and hKNL-2, activators of CENP-A assembly, to centromeres prior to the arrival of nascent CENP-A. In addition, we show that G2 phase loading depends on these factors as well as on the CENP-A chaperone HJURP indicating that CENP-A loading occurs though the canonical pathway normally operating in G1 phase. We hypothesize that during G2 and M phases an inhibitory CDK dependent
phosphorylation step prevents CENP-A from being assembled and that loss of CDK activity in early G1 alleviates this inhibition allowing CENP-A assembly to occur.

1454/B612
Coupling of Isolated Native Kinetochores to Dynamic Microtubule Tips Studied In Vitro.
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Accurate mitosis depends on kinetochores, large protein complexes that link chromosomes to the mitotic spindle. The most fundamental property of kinetochores-their ability to form load-bearing attachments to assembling and disassembling microtubule tips-can be reconstituted In Vitro using individual kinetochore subcomplexes alone, such as Ndc80, Dam1, etc. In vivo, these subcomplexes are thought to act cooperatively, interacting in ways that enhance the coupling performance of the whole assembly; but reconstituted assays using whole kinetochores have not been possible due to the lack of a robust kinetochore isolation method. To study how interactions between subcomplexes affect kinetochore function, we developed a method to isolate native kinetochores (nKTs) from budding yeast via affinity purification of the Mtw1 subcomplex. The pure nKTs retain a majority of kinetochore proteins, including DNA- and microtubule-binding components, and they bind microtubules in a manner that depends on core subcomplexes (e.g., Ndc80 and Spc105). The nKTs also form very long-lived attachments to assembling and disassembling tips that persist for 20 minutes under as much as 6 pN of tensile force, here applied with a servo-controlled optical trap. This coupling performance is far better than that of individual subcomplexes alone, suggesting that cooperative interactions between kinetochore components do make a major contribution to kinetochore-microtubule coupling.

1455/B613
Phosphoregulation of MPS1 Kinase Activity.
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MPS1 is a protein kinase involved in accurate chromosome segregation from yeast to human. It is essential for chromosome congression and signaling of misaligned chromosomes (kinetochore-dependent mitotic checkpoint). MPS1 becomes hyperphosphorylated and hyperactive in mitosis, but the mechanisms regulating its activity are unclear. We identified eleven conserved phosphorylation sites by mass spectrometry on Xenopus MPS1. Single point mutations of each of these sites showed that three of them (P2, P4 and P10) are essential for MPS1 kinase activity and checkpoint signaling. Two of these sites - P10 (T697) and P4 (T707) - are located within MPS1 kinase domain. Phosphorylation-mimicking mutations at these sites could not restore full MPS1 kinase activity or function. On the contrary, phosphorylation at P2 site is efficiently mimicked by acidic amino acids. Interestingly, this site is located outside of the kinase domain, and could provide an invaluable tool to understand the role of MPS1 non-catalytic domain. We are now investigating 1) which kinase is responsible for the phosphorylation at P2 site, 2) how this phosphorylation affects the kinetochore recruitment of MPS1 and other mitotic checkpoint players, and 3) how this phosphorylation regulates MPS1 kinase activity and/or substrate recognition.

1456/B614
Kinetochore Protein Architecture: Protein Copy Number per Kinetochore Microtubule in Vertebrates.
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We have used two-color fluorescence light microscopy methods to obtain nm scale measurements of the relative positions of different kinetochore proteins along the axis of attached kinetochore microtubules (kMTs) at metaphase for both budding yeast (Joglekar et al., Curr. Biol., 2009) and human cells (Wan et al., Cell, 2009). Equally important for understanding protein architecture and function within the kinetochore is the copy number per kMT. Of particular interest are three highly conserved protein complexes (the KMN Network) that assemble stably within the outer kinetochore to produce core attachment sites for kMTs: hKnl1(hBlinkin)/scSpc105; the Mis12 complex of four proteins and the 4-subunit Ndc80 complex (Ndc80(hHec1), Nuf2, Spc24 and Spc25). We have previously developed a fluorescence ratio method using GFP-fusion proteins expressed from endogenous promotors in budding yeast (Joglekar et al, Nat. Cell Biol., 2006) and fission yeast (Joglekar et al., J.Cell Biol, 2008) to measure protein copy number per kMT. for the KMN network, the numbers for both yeast species at metaphase are between 6-8. Here we have used the number of GFP-Nuf2 molecules within a cluster of 16 kinetochores at mid-anaphase of budding yeast (7x16= 112) as a reference for counting GFP-protein number at kinetochores of chicken cells. In the chicken DT-40 cell lines, the endogenous kinetochore protein was knocked out and replaced by the protein fused to GFP expressed either from its endogenous promoter or form another promoter at a low level (Mikami et al., Mol Cell Biol., 2005). Measurements so far indicate for the KMN network of proteins, protein copy numbers ranging from 35 - 60 per kinetochore at metaphase. Serial section analysis by electron microscopy has found a wide variation in metaphase numbers of kMTs (3-7) with an average of 4.3 (Ribeiro et al., Mol Biol Cell., 2009). This yields a copy number of 8-12 per kMT based on our current measurements, a number very similar to those measured for both budding and fission yeast.

Synapse Formation and Function (1457 – 1477)

1457/B615
Functional Marker Proteins in the Mammalian Growth Cone.
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Identification of proteins in the mammalian growth cone has the potential to advance our understanding of this critical regulator of neuronal growth and formation of neural circuit; however, to date, only one growth cone marker protein, GAP-43, has been reported. In the case of adult synapses, a large number of marker proteins localized to various sub-locations in the synapse are known. Here, we successfully used a proteomic approach to identify 945 proteins present in developing rat forebrain growth cones, including highly abundant, membrane-associated and actin-associated proteins. Almost one hundred of the proteins appear to be highly enriched in the growth cone, as determined by quantitative immunostaining, and for 17 proteins, the results of RNAi suggest a role in axon growth. Most of the proteins we identified have not previously been implicated in axon growth and thus, their identification presents a significant first step forward, providing new marker proteins and candidate neuronal growth-associated proteins (nGAPs).

1458/B616
Real-Time Imaging of the Dynamics of Dense-Core Granules at Presynaptic Sites in Hippocampal Neurons.
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The formation of long-term memories requires plasticity of synapses, sites of communication between neurons. Proteins that function as neuromodulators are crucial mediators of synaptic plasticity during memory formation in the hippocampus, and dysfunction in the release of
neuromodulators is known to cause memory deficits in humans. Past work in our lab established that several key neuromodulators synthesized by hippocampal neurons are copackaged in dense-core granules (DCGs) and are cotransported to postsynaptic sites where they undergo regulated release. Work by other research groups suggests that the early phases of memory formation may also require release of key neuromodulators from presynaptic sites. Motivated by these latter results, we assessed if DCGs containing the neuromodulators pro-brain derived neurotrophic factor (proBDNF) and tissue plasminogen activator (tPA) localize to presynaptic sites by using fluorescent chimeras, a triple-label analysis and fluorescence microscopy. Our results indicate that DCGs containing these key neuromodulators localize adjacent to and within the active zones of presynaptic sites. If DCGs containing these neuromodulators are recruited to presynaptic sites because their cargo is released presynaptically, they would be expected to be retained at these sites and exhibit limited mobility. To evaluate DCGs mobility, we conducted a quantitative analysis of trajectories generated by presynaptically localized DCGs and found that the dynamics of seventy-five percent of DCGs at presynaptic sites fit a classic diffusive model and that these DCGs were either immobile or underwent slow, diffusive motion. Fifteen percent of the remaining presynaptically localized DCGs were found to exhibit slow, anomalous diffusion. These findings indicate that DCGs containing neuromodulatory proteins, like synaptic vesicles, are retained at presynaptic sites and would be strategically positioned to undergo release in response to appropriate stimulatory cues. This work was supported by National Institutes of Health grants 2 R15 NS40425-02 and 2 R15 GM061539-02.

1459/B617
Coxsackievirus and Adenovirus Receptor-mediated localization of Acid Sensing Ion Channel 3.
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The Coxsackie and Adenovirus Receptor (CAR) is a developmentally essential protein that is both a viral receptor and an adhesion protein. Emerging evidence suggests the importance of CAR lies in its ability to traffic proteins within heart, epithelial and neuronal cell types. The Acid Sensing Ion Channel 3 (ASIC3) is a H+−gated cation channel primarily expressed in sensory neurons where it is thought to transduce sensory stimuli. Both CAR and ASIC3 interact with the PDZ domain-containing scaffolding protein Postsynaptic Density Protein-95 (PSD-95). Whereas CAR mediates cell surface and junctional localization of PSD-95, PSD-95 increases retention of ASIC3 within the cell resulting in decreased ASIC3 channel activity. We hypothesized that the localization and function of either ASIC3 or CAR would be altered when co-expressed in the presence of PSD-95. COS7 cells were co-transfected with expression plasmids and evaluated for interaction by co-immunoprecipitation, co-localization by immunocytochemistry, and function by electrophysiology. No direct interaction could be detected between CAR and ASIC3. In contrast, CAR, ASIC3 and PSD-95 made a complex identified by triple co-immunoprecipitation. Moreover, triple co-expression resulted in junctional localization of ASIC3 and reversed the decreased current phenotype of ASIC3-PSD-95 co-expression. The interaction and resulting function was ablated when the PDZ-binding domain of either CAR or ASIC3 was mutated, indicating that a PDZ-based interaction with PSD-95 is essential. Although the physiological relevance of this interaction is currently being investigated in neuronal cell models, this data shows that via PDZ-based interactions, CAR can interact with important channels such as ASIC3, and hence may mediate junctional or potentially synaptic localization.

1460/B618
Synaptophysin Targeting in Photoreceptors.
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Photoreceptors are specialized neurons, consisting of several discrete compartments organized in a linear array. These compartments include the photosensitive outer segment, the biosynthetic
inner segment, the nuclear region, and the synaptic terminal which contains ribbon synapses that mediate the tonic release of glutamate in the dark. The signals and mechanisms that ensure the proper sorting, targeting and trafficking of proteins to these various compartments are poorly understood, particularly in the case of the synaptic terminal. The objective of the current study was to determine the minimal sequence within the synaptic vesicle marker protein, synaptophysin, that signals the protein to be targeted to the synaptic terminal. Synaptophysin spans the membrane four times and is found in oligomers within the synaptic vesicle membrane, where it is believed to contribute to the regulation of various aspects of the synaptic vesicle cycle. Our approach was to generate constructs consisting of a membrane-associated YFP as a reporter fused to various regions of synaptophysin, and express those constructs in the rod photoreceptors of transgenic *Xenopus laevis*. The subcellular localization of these proteins in tadpole photoreceptors was assessed by confocal microscopy. The YFP reporter was found throughout the cell but strongly accumulated in the outer segment, as we have previously described for membrane proteins lacking targeting information in this cell type. We found that the cytoplasmic C-terminus of synaptophysin, but not the N-terminus or the intracellular loop region, fused to the reporter was restricted to the inner segment plasma membrane and synaptic terminal. Several deletion constructs of the C-terminus revealed that the last 10 residues of synaptophysin’s C-terminus were sufficient to target the reporter to the synaptic terminal. We conclude that the C-terminal 10 amino acids of synaptophysin contain a targeting signal that directs the subcellular localization of this protein in photoreceptors.

1461/B619

Second Messenger Dynamics and Interactions during Spinal Guidance.

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Cellular second messengers, particularly cyclic adenosine monophosphate (cAMP) and calcium, are critical for axonal pathfinding regulation. However, the dynamics and subcellular localization of cAMP and calcium responses to axon guidance cues are largely unknown in growth cones. We investigated the temporal and spatial structure of second messenger signals in growth cones of cultured embryonic Xenopus spinal neurons stimulated by a local application of Netrin-1. We first monitored cAMP signals and calcium transients in filopodia. Netrin-1 induces a transient increase in cAMP concentration in filopodia. The frequency of filopodial calcium transients follows a similar pattern: it rises for a brief period after the onset of Netrin-1 stimulation. Filopodial calcium transients require cAMP, and the Netrin-1-induced cAMP elevation occurs independently of calcium. Global second messenger signals in spinal growth cones are also affected by Netrin-1. cAMP concentration rises transiently in the center of the growth cones after local Netrin-1 application, and this signal is dependent on calcium signaling. The frequency of global growth cone calcium transients increases dramatically and sustainably after the onset of Netrin-1 stimulation. The global calcium transients are not affected by modifications of cAMP concentration. To evaluate the effect of transient cAMP signals on growth cone behavior, we induced a brief and asymmetric elevation in cAMP concentration across the growth cone. The axon was attracted towards the side with the higher concentration, demonstrating that a transient cAMP signal is able to change the direction of axon outgrowth. Because the influence of cAMP on spinal commissural axons trajectory is still unclear in vivo, we developed a preparation of the Xenopus spinal cord allowing us to image midline crossing by commissural axons while modifying cAMP concentration. Increases or decreases in cAMP level affected commissural axons trajectory In Vivo in a similar way: preventing the crossing of commissural axons. Our results suggest that temporal limitation and/or spatial restriction of cAMP signaling is required for appropriate axon pathfinding by spinal commissural axons. Supported by NIH NINDS 15918.

1462/B620

Connexin43 and Translocator Protein in Rat Brain and Heart Mitochondria are Involved in Carbenoxolone-Promoted Permeability Transition Pore: Control of Cell Death Mechanisms in Cellular Senescence.
It has been reported that connexin (Cx)43 plays a critical role in protection during ischaemic and pharmacological cardiac preconditioning and that this role is independent from gap junction-mediated communication. Carbenoxolone (Cbx) was reported to induce permeability transition pore (PTP) opening in isolated rat liver mitochondria (RLM), although the mitochondrial molecular target of Cbx has yet to be identified. Here, we investigated the effects of Cbx on Ca2+-induced PTP opening in RLM, rat heart (RHM), and rat brain (RBM) mitochondria. In RLM, RHM, and RBM, threshold Ca2+ load induced PTP opening, as seen by sudden Ca2+ efflux from the mitochondrial matrix and a drop in membrane potential. At about 1 μM, Cbx enhanced Ca2+-induced CyclosporinA-sensitive PTP opening. Since in isolated heart mitochondria Connexin43 (Cx43) was found, we suggested that Cbx might be target for Cx43 in mitochondria. We detected Cx43 by Western blot in the outer RBM membrane and in mitoplasts, but not in RLM. Anti-Cx43 antibody abolished Cbx-enhanced PTP opening in RBM but not in RLM, supposing possible involvement of another connexin type in RLM. Besides being a gap junction inhibitor, Cbx bears structural similarity to corticosteroids, indicating possible translocator protein (TSPO)/peripheral-type benzodiazepine receptor (PBR) targeting. Cbx-enhanced PTP opening, which correlated with increased protein phosphorylation, was suppressed by an anti-TSPO antibody. All these results indicate that Cbx facilitates Ca2+-induced PTP opening having as possible targets in RHM and RBM both TSPO and Cx43. Identification of new functions of Cbx and Cx43 in mitochondria of neurons and astrocytes, as reported here, may be relevant for understanding of role of this protein in modulation of cell death and dysfunction in different neuropathological conditions and in ageing-related neurodegenerative mechanisms.

1463/B621
Rapid Wallerian Degeneration is Required for Successful Reinnervation of the Skin by Developing Peripheral Sensory Axons.
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Following injury to peripheral sensory axons, the distal portion of the axon that is detached from the soma breaks down by the process of Wallerian degeneration (WD). The rapid nature of fragmentation and debris clearance implies that genetic pathways or external influences are involved. We have developed a zebrafish model to study sensory axon degeneration after injury in real time in vivo. By combining precise laser axotomy of single axons in the skin with time-lapse confocal imaging, we have 1) characterized the cellular dynamics of degeneration, 2) begun to dissect the roles of intrinsic signaling pathways and extrinsic cell types, and 3) identified regeneration defects in cells with delayed axon degeneration. In wildtype animals, axon degeneration occurred in two stereotyped phases. A "lag" phase, during which the severed axon persisted despite detachment from the soma, precedes a "fragmentation" phase, marked by breakdown of the axon and debris clearance. The lag phase lasted ~2 hrs early in development and ~1 hr at later stages; the fragmentation phase lasted ~1 hr at all stages examined. Both the UPS and NAD pathways regulate the length of the lag phase. In other systems, upregulating NAD by overexpressing the mouse WD slow (wldS) gene leads to axonal protection. Similarly, misexpressing WLDS in zebrafish sensory neurons robustly lengthened the lag phase to at least 48 hrs. Expressing a ubiquitin protease in severed axons also extended the lag phase, but not the fragmentation phase. To identify the phagocytes responsible for removing axonal debris we examined degeneration in fish lacking either macrophages or peripheral glia. Surprisingly, degeneration was not affected in either case, indicating that neither macrophages nor glia are required. We are now testing the hypothesis that epidermal cells are responsible for engulfing axonal debris. Finally, we took advantage of the longevity of WLDS overexpressing axon fragments to assess the consequences of a failure of WD on regeneration of proximal axons. We found that regenerating axons in the skin avoid persistent axon fragments. Thus, rapid clearance...
of degenerating axons during development is required for comprehensive innervation of the epidermis.

1464/B622
The Adaptor Protein APPL1 is a Critical Regulator of Dendritic Spine and Synapse Formation in Hippocampal Neurons.
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The formation and plasticity of dendritic spines and synapses, which are poorly understood on a molecular level, are critical for cognitive functions, such as learning and memory. Adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1 (APPL1) is emerging as a critical regulator of various cellular processes in non-neuronal cells, but its function in the nervous system is not well understood. APPL1 mediates its function through the interaction of important signaling proteins, such as the serine/threonine kinase Akt. In this study, we show that APPL1 is highly expressed in brain and localizes to dendritic spines and synapses in hippocampal neurons. Knockdown of endogenous APPL1 using siRNA led to a significant decrease in the number of dendritic spines and synapses while expression of GFP-APPL1 resulted in a 1.5-fold increase in the density of spines and synapses. The defect in spine and synapse formation, resulting from knockdown of endogenous APPL1, can be rescued by exogenous expression of APPL1, indicating the effects on spines and synapses are due to the loss of the endogenous molecule. Since the APPL1 binding protein Akt is activated downstream of phosphatidylinositol 3-kinase (PI3K), we inhibited PI3K and examined the effect on spines and synapses. Treatment of neurons with the PI3K-specific inhibitor LY294002 abolished the APPL1-promoted increase in the number of spines and synapses. Similar results were obtained with another PI3K inhibitor, wortmannin, suggesting the regulation of spines and synapses by APPL1 is PI3K dependent. Expression of APPL1 lacking its C-terminal PTB domain, which binds Akt, resulted in a significant decrease in the number of dendritic spines and synapses, suggesting Akt is an effector of APPL1 in regulating spine and synapse formation. Consistent with this, expression of APPL1 significantly increased the amount of active Akt in dendritic spines and synapses. In addition, knockdown of Akt with siRNA or expression of dominant negative Akt abolished the APPL1-promoted increase in the number of spines and synapses. Collectively, our results point to a new role for APPL1 and Akt in regulating spine and synapse formation.

1465/B623
The BTB-Kelch Protein KLEIP Controls the Degradation of PDZ-RhoGEF to Regulate Neuronal Morphogenesis.
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The induction of neurite extension is involved in developmental and regenerative processes. Here we identify a BTB-kelch protein KLEIP as a novel regulator of neurite extension. KLEIP is distributed throughout the cell body and neurites, with a higher expression level in the distal than proximal regions of axon. In both neuronal cell line and primary neurons, KLEIP elicits a profound effect on promoting neurite extension, which is dependent on its association with Cul3 to form an ubiquitin ligase complex. PDZ-RhoGEF, a protein highly expressed in brain, binds to the substrate-recognition domain of KLEIP and is ubiquitinated both In Vitro and In Vivo by the Cul3/KLEIP complex. As a result, the KLEIP-containing ubiquitin ligase promotes the proteasomal degradation of PDZ-RhoGEF, thus leading to Rho inactivation. We present evidence showing that this KLEIP-induced PDZ-RhoGEF degradation contributes to the spreading of growth cone and neurite outgrowth effect of KLEIP. Upon activation of the neurite retraction signal transmitted by Ga12/13, however, this KLEIP-mediated PDZ-RhoGEF ubiquitination is suppressed due to the competition of Ga12/13 with KLEIP for PDZ-RhoGEF binding. Our findings indicate that KLEIP
functions as a substrate adaptor of Cul3 ubiquitin ligase to facilitate the proteolysis of a neurite extension inhibitor PDZ-RhoGEF, and uncover an inhibitory mechanism for this PDZ-RhoGEF degradation pathway during certain neurite retraction conditions.

1466/B624

C. elegans, VSM-1, A Negative Regulator of Synaptic Function.
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Exocytosis is a fundamental mechanism employed by eukaryotic cells for the controlled secretion of substances. In the nervous system, exocytosis mediates synaptic vesicle fusion and neurotransmitter release from nerve terminals. The SNARE proteins play a central role in this process. Assembly of these proteins into a ternary SNARE complex is thought to be essential for vesicle fusion in vitro. Progress has been made in identifying the fusion machinery, but the mode of action of SNARE interacting proteins is controversial. Therefore, the focus of our project was to determine the role of C. elegans v-SNARE master protein 1 (VSM-1) in vesicle fusion at the synapse. To this end, we began characterizing the phenotype of vsm-1(ok1468) deletion mutants isolated by the C. elegans gene knockout consortium. This mutant synaptic phenotype was studied using molecular, behavioral, pharmacological, and cytoarchitectural assays. First, molecular analysis of vsm-1(ok1468) mutant transcripts showed the vsm-1 deletion does not fully knock out vsm-1 gene products; in contrast it results in smaller mRNA for vsm-1 and a truncate protein. Second, quantitative analysis of vsm-1(ok1468) locomotion behavior showed that musculature performance is impaired in vsm-1(ok1468) mutants. Third, pharmacological assays demonstrate that vsm-1(ok1468) mutants have a synaptic phenotype characterized by enhanced sensitivity to “Aldicarb,” a cholinesterase inhibitor, while the sensitivity to the nicotinic receptor agonist “Levamisole” is normal. Lastly, immunological analysis of synapses showed vsm1-ok1468 has abnormal synaptic connectivity. These data suggest that C. elegans VSM-1, has an inhibitory role in vesicle exocytosis affecting synaptogenesis and/or synaptic transmission. Specifically, analyses of vsm-1(ok1468) mutants demonstrate that these animals have enhanced neurotransmission and abnormal synaptic network.

1467/B625

Brain-Enriched Na+/H+ Exchanger NHE5 Controls pH at Excitatory Synapses.
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Synapses must maintain homeostasis of multiple parameters such as Ca++ concentration and membrane traffic in order to control normal synaptic transmission. It has been well documented that many components of the synaptic machinery are sensitive to changes in pH within the physiological range. For example both voltage gated Ca++ channels and N-Methyl-D-Aspartate (NMDA) receptors have been shown to be inhibited by small increases in extracellular protons. However, to date very little is known as to the mechanisms which regulate synaptic pH or the molecular identity of the synaptic pH-regulating proteins. We investigated the possibility the Na+/H+ Exchanger 5 (NHE5) may play a role in regulating synaptic pH. NHE5 mRNA is known to be expressed almost exclusively in brain. We show for the first time that NHE5 protein is expressed in neurons of the hippocampus where it is localized in both axons and dendrites as shown by colocalization with the markers Tau and MAP2 respectively. In non-neuronal cells, exogenously-expressed NHE5 was found previously to undergo dynamic trafficking between recycling endosomes and the plasma membrane. We show here that in neurons NHE5 is found in the endosomal compartment, in dendritic spines and at excitatory synapses. We propose a model in which NHE5 traffics between the endosome compartments and the surface of synapses serving as a mechanism to regulate synaptic pH, and consequently, synaptic transmission.
**1468/B626**

**Directional Guidance of Neuronal Growth Cones Using an Optically Driven Micro-motor.**

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**BACKGROUND:** Physical methods of directing axonal growth offer great promise for treating nerve injuries and investigating axonal guidance. Unlike molecular methods acting through receptor systems specific to each neuron, they can work on all neurons. Relatively little effort has been invested in physical methods. Here we report a novel physical method, an optically driven micromotor, that can induce turning movements in growing axons. METHODS: The device consists of a birefringent calcite particle several microns in diameter that was trapped with a circularly polarized laser beam which causes the particle to rotate. The direction of rotation could be controlled by the direction of polarization and the speed of rotation could be regulated by the laser power. RESULTS: In tissue culture, this device generated a controlled microfluidic flow that could be selectively localized to the axonal growth cone. Its effectiveness was tested on optic axons growing from goldfish retinal explants. The velocity of rotation, the time of rotation, the size of the motor and the position and distance from the growth cone were systematically varied. A non-rotating particle did not significantly alter the direction of axonal growth regardless of its position. However, a rotating particle caused growth cone turning and could do so at different speeds of rotation, with different sized particles and at different angular positions relative to the growth cone. Remarkably, when placed in front of the growth cone, the direction of rotation altered the direction of growth. from a fixed position the micromotor could produce a left or right turn depending on clockwise or counterclockwise movement. Previous methods of directing axonal growth from a fixed position including molecular methods have been limited to inducing attraction or repulsion. To our knowledge, this is the first example of a true, left-right turn signal.

**1469/B627**

**Pre-synaptic Active Zone Protein Piccolo Promotes Actin Assembly at the Synapse in Association with Daam1.**

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Piccolo (PCLO), a structural component of the Pre-synaptic Active zone harbors several protein interaction domains and potentially acts as a scaffolding protein at the pre-synapse. PCLO negatively regulates synaptic vesicle exocytosis by regulating Actin dynamics at the pre-synapse. In order to gain further understanding into mechanisms of PCLO mediated Actin regulation we carried out immunoprecipitation assays from brain to identify proteins that associate with PCLO and are also involved in Actin regulation. We identified Daam1 as a novel interacting partner of PCLO. Daam1 is a signaling molecule capable of being regulated by Rho GTPases as well as Wnt signaling protein Disheveled and belongs to the Formin family of Actin polymerizing proteins. We hypothesized that by regulating Daam1 activity, PCLO could regulate Actin. To test this hypothesis we have developed a cell-based Actin polymerization assay and using this assay have established that central region of PCLO can act as a platform for Actin polymerization once associated with Daam1. Co-localization of Daam1 with PCLO in growth cones and at synapses in cultured hippocampal neurons further supports the physiological relevance of this interaction. Daam1 is an adapter molecule capable of linking non-canonical Wnt signaling with Rho-GTPase associated Actin cytoskeleton changes. By binding to Daam1 PCLO may help localize Daam1 activity at certain regions and or in signal dependent situations e.g. signal dependent growth cone steering and/or presynaptic Actin regulation.

**1470/B628**

**The Regulation of Cofilin-Mediated Dendritic Spine Remodeling in Hippocampal Neurons.**
Dendritic spines are the postsynaptic sites of most excitatory synapses in the brain. Cell surface receptors such as EphB and N-methyl-D-Aspartate (NMDA) receptors can initiate intracellular signaling cascades that regulate the stabilization and maturation, or the destabilization and remodeling of these actin-rich structures (Ethell and Pasquale, 2005). The F-actin-severing protein cofilin can induce reorganization and remodeling of mature dendritic spines in cultured hippocampal neurons and its activity is regulated by phosphorylation. We have shown that suppression of this activity promotes mature dendritic spines and synapses, and this can be achieved through an EphB receptor-Focal Adhesion Kinase (FAK) pathway. Inhibition of EphB receptor activity with dominant-negative EphB2 leads to disruption of mature dendritic spines, which can be rescued by overexpression of a constitutively-active form of the immediate downstream factor FAK (Shi et al., 2009). In addition, spine remodeling induced by dominant-negative EphB2 is blocked by overexpression of dominant-negative phospho-mimetic cofilinS3D, but not non-phosporylatable constitutively-active cofilinS3A. The inactive cofilinS3D also rescues dendritic spine phenotype disrupted by Cre-mediated knock-out of FAK. Our studies suggest that EphB receptors promote mature dendritic spines and synapses through down-regulation of cofilin activity. Neuronal activity also regulates cofilin activity through the NMDA receptor and its variety of downstream pathways that can lead to activation or inactivation of cofilin. In addition, NMDAR-mediated Ca2+ currents are potentiated by EphB2 receptor activation of Src (Takasu et al., 2002), which phosphorylates NMDA receptors and increases gating (Salter and Kalia, 2004). Future studies will examine a relationship between activity-dependent and EphB receptor-mediated regulation of cofilin in dendritic spines and their roles in dendritic spine stabilization and remodeling. This work was supported by National Institute of Mental Health Grant MH67121

1471/B629
Regulation of Alpha-synuclein in Mouse Model of Down Syndrome.
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We have demonstrated significant increase in alpha-synuclein expression in adult Ts65Dn cortex. mRNA dependent RT-PCR analyses and protein measurements showed similar increase of 2-3 fold respectively suggesting that alpha-synuclein is regulated at the transcriptional level. Alpha-synuclein is a 140 amino acid protein; mutation/s in this protein shown to be familial in Parkinson disease. Alpha-synuclein is expressed abundantly in the CNS and is involved in a variety of presynaptic functions including endocytosis. Alpha-synuclein is encoded by a gene whose copy number is unchanged in Down syndrome. Suppressed synaptic transmission is one of reasons for deficient mental functions and retardation observed in down syndrome. Immunohistochemical analyses of Ts65Dn currently in progress might provide insight on the role of alpha-synuclein in presynaptic dysfunctions in Ts65Dn brain.

1472/B630
Myosin-dependent Contractility and Actin Bundling Dictates Spine Dynamics and Organizes the Post-Synaptic density.
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The actin cytoskeleton is thought to dictate the morphology and dynamics of dendritic spines and may also organize the post synaptic density, all of which are associated with learning and memory. Dynamic remodeling of actin occurs through its polymerization, depolymerization, crosslinking, and contraction. Non-muscle Myosin IIB (NMII-B) is a major actin associated protein enriched within dendritic spines. NMII-B oligomerizes and organizes actin into thick bundles through its direct actin cross-linking and contractile activities. To parse these contributions, we
generated an ATPase-deficient mutant of NMII-B, R709C, which binds and cross-links, but cannot contract, actin. Expression of this mutant drastically increased spine length when compared to neurons expressing wild-type NMII-B. Ectopic expression of other actin cross-linkers, e.g., α-actinin-1 and filamin B, also increased spine length. In contrast, expression of a constitutively active regulatory light chain mutant, RLC-DD, which increases myosin contractile activity, induced shorter spine length. While actin-crosslinking activities promote spine growth, contractility is required for chemically-induced morphological maturation of the spine, which is characterized by shortening of spine length and concomitant expansion of the spine head. To further assess the role of NMII-B oligomerization in organizing actin into bundles within the spine, we have made a series of phosphomimetic and unphosphorylatable mutations in the C-terminal tail region that controls NMII-B heavy chain oligomerization and actomyosin filament assembly. Finally, we found that NMII-B-mediated contractility organizes the post-synaptic density within the spine. These observations lead us to propose a model whereby a balance between actin bundling and contraction determines spine length. Our model also proposes that NMII-B-induced contractility retracts the spine and pulls on actin tethered to the post-synaptic density in response to NMDA receptor activation, via chemical stimulation, to aid in spine-head expansion during maturation.

1473/B631
Glutamate-induced Cofilin-actin Rod Formation Requires AMPA Receptors and is Associated with a Disruption of APP-YFP Transport.
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Alzheimer disease (AD) pathology is characterized by disruptions to both the actin and microtubule cytoskeletons, which can manifest as cofilin-actin rod-shaped aggregates or neurofibrillary tangles, respectively. Using glutamate excitotoxicity as a model system for cofilinopathies observed in AD, we have determined signaling mechanisms for glutamate-induced cofilin-actin rod formation in young hippocampal neurons. Our results demonstrate that glutamate-mediated rod formation depends on AMPA receptors but not on Ca2+ or the Ca2+-dependent proteins, calcineurin and calpain. Glutamate-induced rods are also co-localized with high molecular weight (HMW) isoforms of microtubule-associated protein (MAP)-2, and the presence of rods is associated with a general loss of trafficking of amyloid precursor protein (APP)-YFP-containing vesicles. Our results suggest that the induction of cofilin-actin rods by excitotoxic levels of glutamate may contribute to AD pathology through a general disruption of MAP proteins and normal vesicle trafficking, and may represent an important mechanism for the pathogenesis of sporadic AD. (Supported by NIH grant NS40371)

1474/B632
The Role of Tropomyosins in Neuronal Development and Disease.
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Morphological changes in developing and adult neuronal cells are strongly supported by the underlying cytoskeletal filament system. A dynamic actin cytoskeleton is required to drive neurite formation and synaptogenesis and is essential for neuronal plasticity and processes of learning and memory in the adult brain. Dysfunction of these processes and disruption of the integrity of the cytoskeleton are associated with a number of neurological diseases including Alzheimer's and Parkinson's disease. Revealing the molecular mechanisms that are involved in the regulation of
the actin cytoskeleton is therefore crucial in order to understand synaptic function during both development and disease. We have previously demonstrated that the association of tropomyosins with actin filaments is a defining factor of the dynamic properties and the stability of the actin cytoskeleton. Tropomyosins are a family of proteins with over 40 different isoforms, generated by alternative splicing from four mammalian genes (α-, β-, γ- and δ-tropomyosin). We have also shown that individual tropomyosin isoforms drive different aspects of neuronal morphogenesis via the generation of functionally distinct actin filament populations. Our aim is to define the function of tropomyosins in regulating the actin filament system at the central nervous system (CNS) synapse. Using a recently developed low density culture system of mouse embryonal hippocampal neurons, we have now determined the subcellular localisation of individual tropomyosin gene products in developing neurons. Our results show a segregation of tropomyosin isoforms to different compartments at the CNS synapse with a post-synaptic localisation of γ- and δ-tropomyosin gene products. This supports the hypothesis of a tropomyosin dependent generation of distinct actin filament populations at synaptic structures. We are currently studying changes in function and/or localisation of tropomyosin isoforms at the CNS synapse in neurodegeneration to better understand the pathology of neurodegenerative diseases.

1475/B633
The homeobox Factor Barx2 Regulates Plasticity of Young Myofibers.
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Adult mammalian muscle retains incredible plasticity. It responds to a chronic increase in mechanical load by increasing its mass and can readily regenerate after injury. These growth and repair mechanisms involve the activation of undifferentiated myogenic precursors called satellite cells. In some circumstances, it has been proposed that existing myofibers may also cleave and produce a pool of proliferative cells that can redifferentiate into new fibers. Such myofiber dedifferentiation has been observed in the salamander blastema where it may occur in parallel with satellite cell activation. Moreover, ectopic expression of the homeodomain transcription factor Msx1 in differentiated C2C12 myotubes has been shown to induce their dedifferentiation. While it remains unclear whether dedifferentiation and redifferentiation occurs endogenously in mammalian muscle, there is considerable interest in induced dedifferentiation as a possible regenerative tool. Here we report that ectopic expression of Barx2 in C2C12 myotubes, MyoD-induced C3H10T1/2 myotubes and differentiated primary mouse muscle cells, caused dedifferentiation evinced by cleavage of the syncytium concomitant with down-regulation of muscle differentiation markers. To better understand the mechanism of this phenomenon, we investigated whether young and mature primary myofibers differ in their ability to dedifferentiate. We microinjected Barx2 and control cDNAs into young or mature primary myofibers and followed their fate over several days. We found that injection of Barx2 cDNA into young myotubes induced cleavage, whereas injection of Barx2 cDNA into mature myotubes induced contraction and cell death. All myofibers (young and old) injected with control plasmids retained their integrity. This finding is also supported by time-lapse microscopy data suggesting that young myofibers maintain a high level of plasticity and can be induced to cleave producing cells that can fuse again. Thus our data suggest that only young myofibers can dedifferentiate in response to homeobox over-expression, while mature myofibers appear to have lost this ability.

1476/B634
Functional Studies of Otoferlin and Dysferlin.
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Prelingual hearing loss is a common hereditary disorder, with approximately 1 out of every 1000 children suffering from profound deafness. Recently, the OTOF gene has been identified as responsible for a type of auditory neuropathy associated with functional deficits downstream of cochlear mechanotransduction. The product of OTOF expression is otoferlin, a transmembrane protein that is hypothesized to act as a catalyst of SNARE mediated neurotransmitter release in the inner hair cells of the cochlea. Otoferlin is believed to represent one member of a mammalian gene family that also includes dysferlin and myoferlin, proteins associated with various forms of muscular dystrophy. All members of this family share a similar fold, consisting of a series of beta sheet C2 domains followed by a single pass transmembrane region. The C2 domain motif is a common feature of lipid membrane binding proteins, including phospholipases, protein kinases, and the synaptotagmin family. However, despite structural similarities to other C2 domain containing proteins, little is known about the functional properties of the C2 domains of otoferlin and dysferlin. Using a set of biophysical assays, we have determined the membrane and SNARE binding abilities of each C2 domain within otoferlin and dysferlin. Further, through use of a SNARE-liposome reconstituted membrane fusion assay, we have determined which C2 domains play an active role in modulating SNARE mediated membrane fusion.

1477/B635
Expression of Exocyst Subunit Exo70 Induces Synapse Formation in Rat Hippocampal Neuronal Cultures.
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Many of the molecular components of the synapse are trafficked to nascent synaptic sites in vesicular assemblies, therefore it is possible that the exocyst could have a significant role during synapse formation at the central nervous system. Here we examine whether the exocyst subunit Exo70 affect synapse formation. for that purpose we expressed Exo70 or the dominant negative N-term domain (N-Exo70) with a bicistronic lentiviral vector using EGFP as a reporter of infected cells. Synapse density on the dendrites of infected cell was evaluated using a Piccolo antibody and FM 4-64 uptake. Expression of Exo70 results in an increased the density of filopodia formation and synapse in the dendrites of infected neurons. Expression of the N-Exo70 did not affect synapse formation in the neurons infected. FM 4-64 uptake experiments suggest that the synapses induced by Exo70 expression are functional. These results suggest that the Exo70 subunit of the exocyst could play a significant role in synapse formation.

Targeting to Lysosomes (1478 – 1488)

1478/B636
Rab-GTPases and Rab-GAPs Involved in Autophagy.
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Objective: to identify Rab-GAPs and their corresponding Rab-GTPase targets involved in autophagy and, in particular, autophagosome formation. Autophagy is a highly conserved bulk degradation process in which double membrane vesicles, called autophagosomes (AVs), form around a portion of cytoplasm or organelles. These AVs fuse with endosomes and lysosomes whereupon they become degradative compartments (AVd). Autophagy is a multi-step process that can be broken down into defined steps: vesicle nucleation, expansion, closure, transport, and, finally, fusion to the endo-lysosomal system. Presumably each step involves a different set of trafficking machinery such as Rab-GTPases, some of which have been linked to autophagy. These are small GTP-binding proteins that perform important functions in membrane trafficking events, mostly by recruiting membrane tethering and fusion machinery. for example, Rab7 has been shown to be essential for the final fusion step, however, little is known about the membrane
trafficking events required for AV nucleation and expansion and the origin of the autophagosomal membrane remains elusive. Results and Conclusion: In order to address these questions, and obtain a comprehensive understanding of the Rab-GTPases involved in the autophagy process, we have systematically screened the Rab-GTPase activating proteins (Rab-GAPs). Our approach was to over-express all 38 putative human Rab-GAPs (characterised by the presence of a TBC-domain) in HEK293A/GFP-LC3 cells thus inactivating their corresponding Rabs. Autophagy induced by starvation, was measured by lipidation of GFP-LC3. Through this screen 12 Rab-GAPs were identified that inhibit autophagy, including TBC1D14. We are now in the process of validating the putative GAPs, and investigating their candidate target Rab proteins and effectors. Special focus will be on TBC1D14, a Rab-GAP that seems to bind to other autophagy-related proteins (Atg proteins).

1479/B637
Mucolipidosis II Mice Accumulate Autolysosomes in Their Serous/Mucous Type Secretory Cells.
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Mucolipidosis type II (MLII) is an autosomal recessive lysosomal storage disorder caused by mutations in the gene encoding the alpha Beta subunits of UDP-GlcNAc:glycosidic enzyme GlcNAc-1-phosphotransferase (GlcNAc-phosphotransferase), the enzyme that catalyzes the first step in the generation of the Man-6-P targeting signal on acid hydrolases. MLII is characterized by skeletal abnormalities, psychomotor retardation, fibroblast inclusions and vacuolization of serous type secretory cells of the pancreas, salivary glands and other tissues1. Recently, mice lacking the alphaBeta subunits of GlcNAc-phosphotransferase (GNPTAB KO) have been generated2. While the fibroblasts lack inclusions, histologic analysis by light microscopy revealed an accumulation of large vacuoles in the serous/mucous type secretory cells. We now report that immunoelectron microscopy of the salivary glands and pancreas shows that these vacuoles are autolysosomes since they are filled with heterogeneous material (including mitochondria and secretory granules) and are LAMP2 positive. Furthermore, we detected an accumulation of the autophagic marker LC3-II by western blotting, and an activation of the secretory granule enzyme trypsinogen in the GNPTAB KO pancreas. Previous studies showed that acid hydrolases enter immature secretory granules (ISGs) and then are retrieved by a Man-6-P dependent pathway and delivered to lysosomes3. In the GNPTAB KO mice, the absence of the Man-6-P signal on the hydrolases would prevent their retrieval from the ISGs, and concomitantly decrease the level of the hydrolases in lysosomes. We propose that an impairment in lysosomal degradation capacity, together with the formation of abnormal secretory granules with premature activation of digestive enzymes results in activation of autophagy in the serous/mucous type cells of the GNPTAB KO mice. 1 Ellender and Martin, 1998. Virchows Arch. 433, 575-578. 2 Gelfman et al., 2007. Invest. Ophthal Mol. Vis. Sci. 48, 5221-5228. 3 Kuliawat et al., 1997. J. Cell Biol. 137, 595-608. *M.B. and E.v.M. contributed equally to this work

1480/B638
Ubiquitination and Ubiquitin Binding by Hua1 Influences the Activity of the E3 Ligase Rsp5.
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Proteins are degraded in a tightly regulated and specific manner to respond to the structural and biochemical demands of the cell. Degradation is initiated by covalent attachment of one or more Ub molecules to the target protein, sometimes referred to as the ‘kiss of death’. Ub attachment is mediated by E3 ligases. The activity of E3 ligases is opposed by the deubiquitinating peptidases (Dubs). These opposing enzymatic reactions are continually at work in order to reinforce or reverse the signal to degrade a particular protein and occur with high specificity. However, it is not known which binding interactions provide the specificity for these reactions. Previously we identified Hua1 as a member of the Rsp5-Ubp2 complex and important for ubiquitination of
proteins at both the TGN and endosomes. Hua1 is a 22kDa protein containing a C-terminal Zn-finger domain of the same subclass as DnaJ proteins and no identified domains within the N-terminus. We have now characterized a novel ubiquitin-binding domain (UBD) within Hua1 both by mutagenesis and NMR structure studies. Studies by others have found that Hua1 is ubiquitinated within this domain. Further, we have identified that this domain of Hua1 and its interaction with Ub is functionally important at multiple locations in the endocytic pathway and provide key interactions to influence the activity of Rsp5 and Ubp2.

1481/B639
Analysis of GGA Null Mice Reveals Distinct Roles for These Adaptors.
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The three homologs of the Golgi-localized, gamma-ear-containing, ADP ribosylation factor-binding proteins (GGAs 1, 2, and 3) of mammalian cells are monomeric clathrin adaptor proteins. Cell-culture based studies showed that GGAs participate in lysosomal enzyme trafficking by packaging mannose 6-phosphate receptors into transport vesicles at the TGN for delivery of their cargo to lysosomes. However, an In Vivo role for the GGAs has not been demonstrated. We now report the consequences of disrupting the genes for GGAs1-3 in mice using insertional mutagenesis. We first determined that the GGAs were expressed at high and equal levels in the brain but GGA2 and GGA3 occurred at extremely low or undetectable levels in other tissues. GGA1 and 3 single null and GGA1/3 double null mice were born in normal Mendelian ratios. While the single null mice maintained viability, the GGA1/3 null mice were small at birth with 50% dying within 24 hours postpartum and another 25% dead within 3 weeks. at 4 months the body weight of the surviving GGA1/3 null mice was reduced 30% compared to wild-type and there was a marked decrease in fat content with the adipocytes having a 3-fold reduction in size. Expression of GGA2, p56 (a GGA accessory protein) and AP-1 in brain was unchanged in all 3 strains. Serum levels of 3 out of 6 lysosomal enzymes were elevated 1.7 to 2.2-fold (p<0.001) in the GGA1/3 null animals, indicating a mild impairment in lysosomal enzyme sorting efficiency that is unlikely to account for the lethality in these mice. Genotyping of 66 pups from a Gga2 +/- cross revealed 15 wild-type, 51 heterozygous and no GGA2 null mice suggesting that inactivation of the Gga2 gene is embryonic lethal. In conclusion, GGAs 1 and 3 have some degree of functional redundancy since loss of either one is well tolerated whereas loss of both results in small offspring with high levels of postpartum death. GGA2 is unable to compensate for loss of both GGA1 and GGA3, suggesting that GGA1/3 perform a distinct function. Similarly, GGA2 loss appears to cause embryonic lethality suggesting a vital function for GGA2 that GGA1 and GGA3 are unable to perform. Together, these data suggest that GGA1/3 and GGA2 have distinct roles.

1482/B640
Extensive Innovation in Rab: Effector Interactions.
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Protein trafficking pathways connect organelles in the endomembrane system, transporting matter and information in a specific and regulated manner. These pathways, with similar molecular components, are found throughout the eukaryotic tree of life and believed to be an ancestral eukaryotic feature. Moreover, different pathways are organized according to the same principles and using similar molecular components, suggesting that they diverged from a common ancestral transport mechanism. RabGTPases are the largest family of trafficking regulators, have been implicated in a variety of functions, such as tethering vesicles to acceptor membranes and linking the vesicles to motor proteins, among others. Rabs are molecular switches that in their active conformation recruit a variety of proteins, Rab effectors, that perform all those functions. We performed the first systematic study on the evolution of Rab effectors in human and in yeast. In contrast to the scenario describe above, we found that Rab effectors rarely arise by duplication, that they are not conserved between orthologous Rabs in yeast and human, and that they appear to emerge in a taxon-specific manner. Analysis of the structural details reveals some commonalities in the interfaces that are likely to have resulted from convergent evolution. Our
results reveal that in a background of a system that evolves by duplication and divergence, critical innovation and specificity is achieved by recruitment of novel components and functionalities. These results further suggest that pharmacological targeting of conserved Rab-mediated mechanisms in human parasites is feasible, as they will involve distinct effectors and interfaces. We made this data available in a publicly available resource - TrafficDB. This resource also contains the mapping of putative effector orthologues across all eukaryotic genomes, as well as an automated classification of Rab proteins in those species.

1483/B641
A Coated Vesicle-Associated Kinase of 104 kDa (CVAK104) Induces Lysosomal Degradation of Frizzled 5 (Fzd5).
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Receptor internalization is recognized as an important mechanism for controlling numerous cell-surface receptors. This event contributes not only to regulate signal transduction, but also to adjust the amount of cell-surface receptors. Frizzleds (Fzds) are seven-pass transmembrane receptor family proteins for Wnt ligands. Recent studies indicated that Fzd5 is internalized in response to Wnt stimulation to activate downstream signaling pathways. After internalization, it appears that Fzd5 is recycled back to the plasma membrane. However, whether internalized Fzd5 is sorted to lysosomes for protein degradation remains unclear. We here report that a coated vesicle-associated kinase of 104 kDa (CVAK104) selectively induces lysosomal degradation of Fzd5. We identify CVAK104 as a novel binding partner of Dishevelled (Dvl), a scaffold protein in the Wnt signaling pathway. Interestingly, we find that CVAK104 also interacts with Fzd5, but not with Fzd1 or Fzd4. CVAK104 selectively induces intracellular accumulation of Fzd5 via the clathrin-mediated pathway, which is suppressed by coexpression of a dominant-negative form of Rab5. Fzd5 is subsequently degraded by a lysosomal pathway. Indeed, knockdown of endogenous CVAK104 by RNA interference (RNAi) results in an increase in the amount of Fzd5. In contrast, Wnt treatment induces Fzd5 internalization but does not stimulate its degradation. Overexpression or knockdown of CVAK104 results in a significant suppression or activation of the Wnt/β-catenin pathway, respectively. These results suggest that CVAK104 regulates the amount of Fzd5 by inducing lysosomal degradation, which probably contributes to the suppression of the Wnt signaling pathway.

1484/B642
A Novel Pathway for GPCR Lysosomal Sorting Independent of Ubiquitination and Ubiquitin-binding ESCRT Subunits.
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Trafficking of G protein-coupled receptors (GPCRs) has critical functions in signal termination and propagation as well as receptor resensitization. However, the molecular mechanisms that control intracellular trafficking of mammalian GPCRs remain poorly understood. Protease-activated receptor-1 (PAR1), a GPCR for the coagulant protease thrombin, is irreversibly proteolytically activated. Consequently, desensitization and endocytic trafficking are critical for regulation of PAR1 signaling. We previously showed that activated PAR1 is internalized, sorted directly to lysosomes and degraded, a process critical for termination of receptor signaling. Moreover, the efficiency with which PARs are degraded makes this receptor class an excellent model system to investigate the molecular basis of GPCR lysosomal sorting. The related PAR2 is modified with ubiquitin, which facilitates lysosomal trafficking through the ubiquitin-dependent endosomal-sorting complex required for transport (ESCRT) pathway. However, activated PAR1 traffics from endosomes to lysosomes independent of ubiquitination and ubiquitin-binding ESCRT components. An ubiquitination-deficient PAR1 mutant degraded comparably to wildtype receptor after activation. Moreover, activated PAR1 sorts to lysosomes independent of the canonical
ubiquitin-binding ESCRT machinery since ablation of HRS (ESCRT-0) and Tsg101 (ESCRT-I) failed to block receptor degradation. Remarkably, siRNA-targeted depletion of Vps4, an AAA-ATPase required for ESCRT function, and co-expression of Vps4-E228Q dominant-negative failed to effect activated PAR1 degradation, whereas agonist-promoted PAR2 degradation was substantially inhibited. We demonstrate that activated PAR1 is nevertheless sorted into intraluminal vesicles (ILVs) of the multi-vesicular bodies (MVBs) prior to degradation. The ESCRT-III complex proteins, termed charged MVB proteins (CHMPs), can facilitate ILV formation independent of other ESCRT proteins. In pilot studies, we found that expression of a CHMP3 dominant-negative mutant inhibits PAR1 degradation. Thus, CHMP3 and other ESCRT proteins required for PAR1 degradation may constitute a novel mechanism for ubiquitin-independent endocytic cargo sorting to the lysosome.

1485/B643
The Molecular Mechanism and Function of prApe1 Aggregation and Atg19 Binding.
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The aggregation of misfolded proteins can interfere with cell function, leading to disease and ageing. In contrast, autophagy enables cells to eliminate and recycle damaged components, and to survive when starved of vital nutrients. The Cytoplasm-to-vacuole-targeting (Cvt) pathway in *Saccharomyces cerevisiae* encompasses both protein aggregation and the autophagic machinery; it involves the aggregation of the premature form of Aminopeptidase 1 (prApe1) in the cytosol, and its subsequent sequestration by autophagic proteins into a vesicle for transport to the vacuole. Although the autophagic proteins involved in the Cvt pathway have been identified through genetic approaches, their function is not fully understood; nor has the molecular mechanism and purpose of prApe1 aggregation been elucidated. We propose that the propeptide of prApe1 is important for aggregation and vesicle formation. This involves the prApe1 receptor, Atg19, which competes against other prApe1 proteins for binding to the propeptide. For our studies we purified and assembled prApe1 and Atg19 aggregates *in vitro*. We are currently expanding these techniques to isolate prApe1 and Atg19 aggregates, as well as autophagic membrane, and thereby form Cvt vesicles *in vitro*. Just as similar In Vitro assays greatly furthered our understanding of intracellular vesicular traffic, this assay will help us elucidate the function of key autophagic proteins.

1486/B644
Characterization of a Novel Beclin Family Member in Autophagy.
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Autophagy is an evolutionarily conserved pathway responsible for clearance of long-lived proteins, damaged organelles, aggregated-prone proteins and invasive pathogens. Double-membrane autophagosomes enwrap cargos and transport them to lysosomes for degradation. However, the molecular mechanisms of autophagy, and its relationship to other lysosomal degradation pathways, are not fully understood in higher eukaryotes. Here, we identified a novel BH3-only protein that shares homology to the autophagy protein Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein), and we designated this protein as Beclin 2 (also called Beclin 1-like protein 1 in human genome databases). We found that Beclin 2 interacts with Bcl-2, as well as several components of the Beclin 1/ class III PI3K complex. siRNA knockdown of Beclin 2 hinders degradation of the autophagic cargo receptor p62 under starvation conditions, suggesting that Beclin 2, like Beclin 1, is essential for autophagy. In addition, through a yeast two-hybrid library screen for Beclin 2-interacting partners, we identified proteins involved in endocytic trafficking of G protein-coupled receptors (GPCR) that specifically bind Beclin 2 but not Beclin 1, indicating that Beclin 2 may also have a function in lysosomal downregulation of GPCR signaling.
Thus, these findings highlight the functional and mechanistic diversity of the Beclin family members in autophagy and endocytosis.

1487/B645

Regulation of Fas Ligand Trafficking and Function in T Lymphocytes.

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Programmed cell death plays a crucial role in cell biological processes such as maintenance of lymphocyte homeostasis, control of autoimmunity, and elimination of infected and malignant cells. Fas ligand (FasL) is a tumor necrosis factor family (TNF) cytokine that induces programmed cell death in a number of cell types through engagement of its receptor Fas/CD95. This interaction induces cell death through recruitment of the adaptor protein FADD and Caspase-8, which initiates a cell death cascade. Fas-FasL interactions are critical in enforcing immunological self-tolerance, since FasL or Fas deficiency on T cells, B cells, or APCs results in autoimmune disease manifestation. In T cells, FasL is synthesized in response to T cell receptor stimulation and induces autocrine cell death through engagement of Fas. Initially synthesized as a transmembrane protein, FasL can be shed from the cell surface via proteolysis by metalloproteases into a soluble form that is considered to be biologically inactive. Alternatively, FasL can traffic to secretory lysosomes from which it is secreted in microvesicles in a membrane-bound form. The relative potency of plasma membrane vs. microvesicular FasL in target cell killing or autocrine cell death is not clear. Three motifs in the intracytoplasmic N-terminal of FasL have been suggested to play a role in directing FasL to secretory lysosomes: N-terminal tyrosines, a proline-rich domain (PRD) that may interact with SH3 domains on other proteins, and two lysines adjacent to the PRD that are ubiquitinated and may tag FasL for recognition by the ESCRT complex of trafficking proteins. Using a panel of FasL mutants in these motifs fused to GFP, we are examining the different motifs that govern FasL trafficking in secretory lysosomes and its secretion in exosomal form in T lymphocytes. Using FADD deficient cells or adding a mutation on FasL that disables its Fas binding ability, we were able to study FasL trafficking in the absence of cell death. Results so far have indicated distinct roles for the PRD and ubiquitination motifs in directing FasL trafficking in T cells. Future studies will investigate the functional consequences of altered FasL trafficking in T lymphocytes.

1488/B646

The HECT Domain of the Ubiquitin Ligase Rsp5 Contributes to Substrate Recognition.

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Objective: Determine the mode of interaction between the ubiquitin ligase Rsp5 and its substrate, Cps1. Results: Ubiquitin modification of endosomal transmembrane proteins plays a significant role in targeting proteins into multivesicular bodies (MVBs) en route to lysosomal degradation. Substrate sorting into MVB intraluminal vesicles is a tightly regulated process. Carboxypeptidase S (Cps1) has served as a model MVB cargo in demonstrating that ubiquitin modification is a positive MVB sorting determinant. Direct interaction between Rsp5 and Cps1 In Vitro has previously been observed, and it has been shown that Cps1 MVB targeting requires the peptide sequence “PVEKAPR”. In order to resolve the mode interaction between Rsp5 and Cps1, we have utilized a variety of In Vitro and In Vivo approaches that have uncovered a novel direct interaction between the HECT domain of Rsp5 and the Cps1 sequence PVEKAPR. Mutations within PVEKAPR perturb the functional interaction between Rsp5 and Cps1 in the absence of Bsd2 both In Vitro and in vivo. Additionally, we observe distinct Cps1 MVB sorting phenotypes upon loss of Bsd2 as compared to loss of Rsp5 function in vivo. Conclusions: These results suggest a model wherein ligase-substrate interactions may be regulated by tertiary factors that modulate their interaction in order to achieve appropriate levels of substrate ubiquitination.
Structure and Function of Membrane Proteins (1489 -1505)

1489/B647
Ric-8B Accelerates \(G_s\) Signaling through the Stabilization of the \(\alpha\) Subunit of Stimulatory \(G\) Protein.
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Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) transmit extracellular signals from G protein-coupled receptor to specific effector proteins. The \(\alpha\) subunit of stimulatory G protein (\(G_\alpha_s\)) is involved in cAMP signaling by activating the adenylyl cyclase, which catalyzes cAMP production, and regulates many physiological aspects such as cardiac regulation and endocrine systems. Ric-8B has been identified as \(G_\alpha_s\)-binding protein; however, its function in \(G_\alpha_s\) and \(G_s\) signaling remains obscure. In this study, we present the evidence that Ric-8B specifically and positively regulates the \(G_s\) signaling by stabilizing \(G_\alpha_s\) protein. In Vitro biochemical study suggests that Ric-8B does not possess the guanine nucleotide exchange factor (GEF) activity. However, knockdown of Ric-8B attenuated \(\beta\)-adrenergic agonist-induced cAMP accumulation, indicating that Ric-8B positively regulates \(G_s\) signaling. Interestingly, overexpression and knockdown of Ric-8B resulted in the increase and decrease of \(G_\alpha_s\) protein, respectively, without affecting \(G_\alpha_s\) mRNA transcription. We found that \(G_\alpha_s\) protein is ubiquitinated, and this ubiquitination is inhibited by Ric-8B. This Ric-8B-mediated inhibition of \(G_\alpha_s\) ubiquitination requires interaction between Ric-8B and \(G_\alpha_s\), because Ric-8B variants, defective for \(G_\alpha_s\) binding, failed to inhibit the ubiquitination of \(G_\alpha_s\). Taken together, these results suggest that Ric-8B plays a critical and specific role in the control of \(G_\alpha_s\) protein levels by modulating \(G_\alpha_s\) ubiquitination, and positively regulates \(G_s\) signaling.

1490/B648
Cell Cycle-regulated Golgi Stack Assembly and Function.
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The molecular mechanism of the Golgi disassembly and reassembly process during the cell cycle of animal cells has been revealed by a defined In Vitro reconstitution assay. Mitotic Golgi fragmentation involves membrane vesiculation coupled with cisternal unstacking; post-mitotic Golgi reassembly is mediated by membrane fusion to form single cisternae and stack formation. Stack formation directly involves the Golgi stacking protein GRASP65 and GRASP55, which play complementary and essential roles in Golgi cisternal stacking by forming mitotically regulated trans-oligomers. By inhibition of GRASP65/55 oligomerization we are able to manipulate Golgi stack formation and thus determine the biological significance of stacking for the first time. We demonstrate that Golgi cisternal unstacking stimulates COPI vesicle budding and protein transport. Golgi fragmentation, however, impairs glycosylation of cell surface proteins and reduces cell adhesion. We propose that Golgi stack formation is a flux regulator for protein trafficking and thereby maintain the quality of protein glycosylation. Structural and functional Golgi defects in disease models are explored in this study.

1491/B649
Two Dimensional Crystallography of the ABC Transporter MsbA.
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MsbA is an ATP-binding cassette (ABC) transporter that exports lipid a and various substrates across the cellular membrane of Gram-negative bacteria. MsbA and related exporters, such as human P-glycoprotein, play a central role in multidrug resistance. Several conformational states of MsbA have now been solved through x-ray crystallography and cryo-electron microscopy. There is, however, a debate about the conformation of membrane embedded MsbA in the
absence of nucleotide. My objective is to use 2D crystallization to study the nucleotide free state of MsbA in a native-like lipid bilayer environment. We have successfully grown crystals of multiple MsbA orthologs by using a variety of methods for reconstitution into membranes. Further refinement of these methods will allow us to produce better diffracting crystals that can be analyzed with programs such as 2dx to generate a projection map of the apo structure of MsbA.

1492/B650
Exploring the Mechanism of Phospholipid Recognition by P4-ATPases.
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In *Saccharomyces cerevisiae*, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and perhaps phosphatidylcholine (PC) are restricted to the cytosolic leaflet of the plasma membrane, while glycosphingolipids are restricted to the extracellular leaflet. This membrane asymmetry appears to be established by P4-ATPases, which are phospholipid translocases (flippases). These pumps couple energy from ATP hydrolysis to movement of a subset of phospholipids from the extracellular/lumenal leaflet to the cytosolic leaflet of the lipid bilayer. The importance of the P4-ATPases is highlighted by their conservation among all eukaryotes and a severe liver disease caused by deficiency in a human P4-ATPase (Atp8b1). To date, the mechanism of substrate recognition by P4-ATPases has not been elucidated. The goal of this work is to determine the mechanism of substrate recognition by defining the important regions/residues for substrate interaction. Drs2 and Dnf1 are *S. cerevisiae* P4-ATPases that differ in their substrate specificities, with Drs2 preferring PS and PE while Dnf1 favors PC and PE. Based on sequence comparison, we hypothesize that the region between transmembrane segments 1 and 2 (lumenal loop 1) is responsible for substrate recognition. A combination of site-directed mutagenesis and overlap extension PCR were used to generate mutants with potential substrate binding domains exchanged between Drs2 and Dnf1. We hoped to convert Dnf1 from a PC/PE flippase to a PS flippase by swapping the potential substrate binding residues in Dnf1 to the analogous residues of Drs2. A set of Dnf1/Drs2 swap proteins have been generated and appear to localize normally. Thus far, certain swap mutations within the target region have abrogated the PC/PE flippase activity of Dnf1, but have not converted Dnf1 to a PS flippase. Other swaps have not affected the flippase activity, but leave the protein unable to complement in vivo. We conclude that lumenal loop 1 is critical for an undefined essential function of P4-ATPases.

1493/B651
Evolutionary Origin of PFUS/PGM Paralogs.
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Parafusin (PFUS) is a paralog of phosphoglucomutase (PGM) with specific functions as a scaffold and signaling protein in exocytosis. PFUS exhibits 50.7% identity to rabbit muscle PGM but has negligible PGM enzymatic activity. In Paramecium, Southern blot analysis reveals four PFUS/PGM genes. We wished to examine the origin of the evolutionary divergence between PFUS and PGM. Database analyses of PFUS/PGM sequences against archa and eubacteria sequences suggest that the divergence seen in Paramecium first occurred in eubacteria, possibly before the origin of the eukaryotic cell. To demonstrate that PFUS is different from other PGM isoforms, endogenous PFUS was immunoprecipitated with pan-PGM antibody and 2D gel analysis was performed. Six spots at Mr 63 kDa with pl’s from 6.8 to 6.3 were detected. Immunoblot analysis using a PFUS specific peptide antibody (I-2) recognized only two spots, pl 6.7 and 6.5; these may represent the two types of PFUS posttranslational modifications. When RNA interference of PFUS is used to knockdown the protein, Western blot analysis showed that the PFUS-immunoreactive band disappears with no obvious effect on that corresponding to PGM. Therefore, PFUS retained its evolutionary distinctness and novel functionality while ciliate protista such as Paramecium evolved by subsequent genome duplications.
1494/B652
Tyrosine and Serine Phosphorylation Regulate the Conformation and Subsequent Threonine Phosphorylation of the L1 Cytoplasmic Domain.
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Previously we identified threonine-1172 (T1172) in the cytoplasmic domain of the cell adhesion molecule L1 (L1-CAM, CD171) as phosphorylated in pancreatic cancer cells. This modification regulated the extracellular conformation, integrin binding, and ADAMs-mediated proteolysis of L1 in these cells. However, although both CKII- and PKC-blockade suppressed this modification in cells, only CKII was capable of phosphorylating T1172 of a recombinant L1 cytoplasmic domain in vitro, suggesting the requirement for additional events to facilitate availability of T1172 to PKC. To study the regulation of this event, we generated a phospho-T1172-specific antibody, and an antibody that recognizes the sequence flanking T1172 independent of the T1172 phosphorylation state. Using recombinant proteins, we demonstrate that the region around T1172 exists in distinct conformations based on both T1172 phosphorylation and the integrity of surrounding residues. We demonstrate that removal of adjacent sequences facilitates T1172 phosphorylation by PKCα specifically, an event that can also be facilitated by prior phosphorylation of serine-1181 by CKII. Importantly, we show that phosphorylation of serine-1181 is required for T1172 phosphorylation by CKII in both the neuronal and nonneuronal isoform of L1. We further demonstrate the role of membrane-proximal and membrane-distal residues in regulating cytoplasmic domain conformation, and that modification of 3 of the 4 tyrosines in the L1 cytoplasmic domain promote conformational changes that facilitate other events. In particular, phenylalanine-substitution of tyrosine-1151 or tyrosine-1229 promote opening up of the cytoplasmic domain in a manner that facilitates phosphorylation of the other 3 tyrosines, as well as phosphorylation of T1172 by PKCα. These data define a specific role for secondary structure in regulating the availability of T1172 that facilitates phosphorylation by PKC and CKII.

1495/B653
Electron Crystallographic Studies of YdhE.
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Multidrug transporters play an essential role in drug resistance in prokaryotes and humans. One family of bacterial multidrug transporters, classified as multidrug and toxic compound extrusion (MATE), include the proteins NorM and YdhE. Studies have shown NorM and YdhE to cause resistance to drugs such as Ciprofloxacin which is used to treat gonorrhea. Little is known about these transporters and to date no structural data exists. The objective of my work is to elucidate the structure of YdhE in context of the membrane. We have successfully grown 2D crystals of lipid reconstituted YdhE. Based on data collected we have generated 2D projection maps of YdhE. The crystals were processed by traditional electron crystallographic techniques and electron tomography including 3D volume averaging. Future studies will examine the relationship between substrates known to bind to YdhE and protein structure/conformation.

1496/B654
Assembly of Caveolin-1 and Cavins during the Caveolar Life-Cycle.
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The integral membrane protein caveolin-1 (CAV1) is the major coat protein of caveolae. Recent studies have identified the cavin protein family members PTRF/cavin-1, SDPR/cavin-2, and SRBC/cavin-3 as additional caveolar components. To dissect the assembly of caveolae and the association of cavins, we followed the fate of newly synthesized CAV1 and cavins biochemically and using fluorescent live-cell microscopy in tissue culture cells. We found that CAV1 homooligomerized into 8S complexes in the ER. After COPII vesicle mediated transport to the Golgi complex, these basic building blocks of the caveolar coat associated with cholesterol and each
other to form homogeneous complexes that sedimented as 70S complexes after delipidation using nonionic detergents. Only after transport of these stable caveolar membrane domains in special Golgi-derived vesicles to the plasma membrane, was PTRF/cavin-1 recruited from a cytoplasmic pool. The late arrival, the relatively slow kinetics (about 15 min), and stable association argue for involvement of PTRF/cavin-1 in maintenance, induction of curvature, budding or other late processes in the caveolar life cycle. Both in the presence and absence of CAV1, the majority of PTRF/cavin-1 in the cell was found in cytosolic complexes sedimenting at 60S. When the other cavin family members SDPR/cavin-2 and SRBC/cavin-3 were individually overexpressed or coexpressed, they were present in the same caveolar adaptor complex. Taken together, we identified novel assembly intermediates of CAV1 and cavins and acquired spatio-temporal information on how cavin proteins associate with newly assembled caveolar domains.

1497/B655
Role of CD38 for cADPR and NAADP Production in Thrombin/ADP-induced Platelet Aggregation.
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CD38 is a multifunctional enzyme, producing potent Ca\(^{2+}\) mobilizers, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP). In this study, we have investigated a role of CD38 in platelet aggregation. Incubation of platelets with physiologic agonists, thrombin/ADP resulted in a rapid rise of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), which is sustained for a long period of time (>10 min), correlating with an increase of platelet aggregation. Thrombin/ADP induced to form cADPR followed by NAADP production through CD38 activation, as platelets prepared from CD38\(^{-/-}\) mice did not produce the messengers and hence no aggregation is achieved by the treatment with thrombin/ADP. Inositol trisphosphate receptor blocker, xestospongin C blocked only the initial phase of thrombin/ADP-induced Ca\(^{2+}\) signal. However, baflomycin, H\(^+\)-ATPase inhibitor, pre-treated platelets showed an abolishment of the late phase of thrombin/ADP-induced Ca\(^{2+}\) signal. Pre-treatment of platelets with an 8-Br-cADPR produced the same pattern of Ca\(^{2+}\) signal as of xestospongin C. Our data showed that Ca\(^{2+}\) signal and aggregation were correlated. Substantial increase in bleeding time was found in the platelets of CD38\(^{-/-}\) mice, compared to the wild type mice. These results demonstrate that CD38 plays an essential role in thrombin/ADP-induced platelet aggregation. Supported by KOSEF grant R0A-2007-000-20121-0.

1498/B656
Caveolin-1 and Sphingolipid GM1 Exhibit Distinct Distributions Among Bovine Rod Outer Segment Disk Membranes.
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Flow cytometry was used to determine the presence of caveolin-1 and the sphingolipid GM1 on bovine rod outer segment disk membranes. First, the feasibility of flow cytometry to detect individual osmotically intact disks was established. Fluorescent beads of 1.0\(\mu\)m, 0.5\(\mu\)m, 0.2\(\mu\)m, and 0.1\(\mu\)m diameter were used to demonstrate that side scatter intensity measured on a log scale corresponds to bead diameter. The intensity of disk side scatter corresponded to 0.1-0.2\(\mu\)m diameter beads. This agrees well with the reported diameter of 0.1-0.5\(\mu\)m for bovine disks [Exp Eye Res. 1975 Mar;20(3):211-7]. We next applied this technique to detect caveolin-1 and GM1, which are present in small quantities in disk membranes. Caveolin-1 was detected with FITC-labeled anti-caveolin-1 and GM1 with FITC-labeled cholera toxin subunit B. Fluorescence due to caveolin-1 binding was detected in 83% of the events that corresponded to scattering attributed to disks. Fluorescence attributed to GM1 was detected in 55% of the events that corresponded to disk scattering. We then determined whether these components are uniformly distributed amongst the disks. The intensity of fluorescence from caveolin-1 labeling was directly proportional to the intensity of side scatter, suggesting that caveolin-1 concentration is uniform amongst all the disks. for GM1 labeling, fluorescence intensity was independent of side scatter.
intensity, suggesting GM1 concentration is not uniform amongst the disks. These results are consistent with caveolin-1, an integral membrane protein remaining in the disk bilayer as the disks are apically displaced. Like other disk lipids GM1 may undergo turnover during apical displacement. GM1 distribution may be similar to the heterogeneous distribution of cholesterol [J Biol Chem. 1990 Nov 5;265(31):18867-70].

1499/B657
Integration of Stimulation of Calcium Sensing Receptor by L-histidine with L-type Voltage Dependent Calcium Channels in Insulin Producing β-cells.
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The objective of this study was to analyze the involvement of L-histidine-induced spatial interaction between a calcium sensing receptor (CaR) and L-type VDCC in controlling Ca\(^{2+}\) channel activity in insulin producing β-cells. Rat insulinoma (RINr1046-38) insulin-producing β-cells were cultured on 25 mm diameter glass coverslips in six-well culture plates in a 5% CO\(_2\) incubator at 37°C. The cell culture medium RPMI-1640 was supplemented with fetal bovine serum, penicillin, and streptomycin. After 5 days of culture, RIN cells were loaded with Fura-2 and the intracellular calcium concentrations, [Ca\(^{2+}\)], were determined by ratio fluorescence microscopy. The additions of increasing concentrations of L-histidine along with 10 mM glucose caused significant changes in [Ca\(^{2+}\)]. To determine the spatial interactions between CaR and L-type VDCC, β-cells were exposed to 2, 5, 10, 15, 20, and 25 mM L-histidine for 15 min at 37°C. The β-cells were then fixed with Bouin and treated with (i) an antibody raised against L-type VDCC, Cav1.2 and (ii) an antibody raised against a peptide mapping within a C-terminal cytoplasmic domain of CaR, followed by treatment with Alexa Fluor coupled fluorescent secondary antibodies. The confocal fluorescence images were acquired on a Nikon TE2000U inverted fluorescence microscope equipped with a Nikon C1 laser scanning confocal microscope system (Nikon Corp., USA). The confocal fluorescence imaging data obtained by using immunofluorescent labeled antibodies against CaR and L-type VDCC showed enhancement in the colocalization correlation coefficient between CaR and VDCC in β-cells exposed to L-histidine thereby indicating increased membrane delimited spatial interactions between these two membrane proteins. The results described in the present study further strengthen that VDCC and CaR can interact spatially to allow control over channel activity through direct protein-protein contact. The L-type VDCC could therefore be potential therapeutic target for prevention of β-cell apoptosis and necrosis during the development of diabetes.

1500/B658
Genome-Wide SGA Screen for V-ATPase Assembly Factors.
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Assembly of the V\(_0\) subcomplex of the V-ATPase within the yeast S. cerevisiae requires five ER-localized chaperone/assembly factors. A genome-wide synthetic genetic array (SGA) screen was performed to identify additional factors that aid in assembly, transport and function of the V-ATPase complex. A strain that is partially compromised for V-ATPase assembly--vma21QQ--was crossed with a haploid genome deletion library. A number of genes emerged as enhancers of the query strain and two interesting candidates genes were chosen for further analysis: FRT1 and ORM2. Both genes have highly similar paralogs, FRT2 and ORM1, respectively. Analysis of FRT1 and FRT2 revealed that whereas the double mutant displayed a synthetic growth defect, the V-ATPase was localized to the vacuole and was fully active. In addition, vma21QQ frt1Δ frt2Δ yeast displayed WT levels of Vph1p, vacuolar acidification, and V-ATPase localization. However, we found that loss of both ORM1 and ORM2 caused a significant reduction in V-ATPase activity. vma21QQ ormt1Δ ormt2Δ yeast cells exhibited a dramatic growth defect on media containing calcium; this was accompanied by a reduction in Vph1p levels, ER-localization of the V\(_0\) subcomplex, and greatly diminished vacular acidification. These results are highly suggestive of
a V-ATPase assembly defect. Both Orm1p and Orm2p are integral ER membrane proteins, and we are investigating whether the Orm proteins physically associate with the V₀ subcomplex during various stages of assembly. Orm1p and Orm2p, which are highly conserved in humans, are thus the newest V-ATPase chaperone/assembly factors to be identified by yeast genetics.

1501/B659
Degradation Mechanisms Responsible for Decreased Levels of a Neutral Endopeptidase (NEP) Variant Allozyme.

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OBJECTIVE: Neutral endopeptidase (NEP) is a type II integral membrane protein that inactivates several important peptides such as natriuretic peptides, glucagon, enkephalins, oxytocin, and bradykinin. We resequenced the NEP gene and found that the NEP variant allozyme, 217A>G (Met73Val) had significantly lower protein and enzymatic activity levels than wild-type (WT). We set out to examine the mechanisms by which degradation of NEP occurs that could account for this variation in enzyme protein levels. METHOD: The variant allozyme of NEP, Val73, and WT were expressed in COS-1 cells. Fluorescence microscopy and quantitative Western blot analysis were performed after treating the cells with either proteasome (MG132) or autophagy (3-methyladenine, 3MA) inhibitors. RESULTS: NEP was localized with the endoplasmic reticulum (ER) and nuclear membrane of COS-1 cells by fluorescence microscopy. Treatment of cells expressing the Val73 variant allozyme with MG132 resulted in a 6-fold increase in immunoreactive variant protein for the variant allozyme. MG132-treated-Val73 cells were also found to have increased fluorescence as compared to non-MG132-treated-Val73 cells, but there were no significant differences between non-MG132-treated-WT and MG132-treated-WT cells, thus demonstrating the important role that proteasomes play in the degradation of the variant allozyme. We also observed increased microaggregate formation with 3MA treatment of cells expressing both WT and Val73 NEP allozyme. However, the number of cells with microaggregates was significantly higher in 3MA-treated-Val73 cells than in WT cells (p ≤ 0.0005), demonstrating the role of autophagy in the degradation of this protein. In addition to these data, we also demonstrated that the chaperone proteins, BIP and GRP94, were involved in Val73 NEP degradation. CONCLUSION: The reduced levels of NEP encoded by a variant allele with the nonsynonymous cSNP 217A>G (Met73Val) is due to rapid degradation by ubiquitin-proteosome dependent processes and by autophagy.

1502/B660
Ancestral Reconstruction of the Yeast V-ATPase Proteolipids.

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The yeast V-ATPase complex is composed of two subcomplexes, the V₁--responsible for ATP hydrolysis--and the V₀, responsible for proton translocation across membranes. There are three integral membrane subunits that comprise the "proteolipid" ring in the fungal V₀ subcomplex: Vma3 (c), Vma11 (c'), and Vma16 (c’). However, virtually all other eukaryotic organisms contain only two proteolipid subunits, orthologs of Vma3 and Vma16. The purpose of our study was to perform a molecular and evolutionary analysis of the V-ATPase proteolipid subunits and assess the unique function of each subunit. Due to their high identity, Vma3 and Vma11 are believed to have arisen from a gene duplication event that can be traced back to the fungal-metazoan split. In order to understand the function of fungal-specific, Vma11p, we used ancestral reconstruction [1] employing a maximum likelihood analysis to create an "ancestral Vma3-11" proteolipid (Anc3-11). The PRANK algorithm was used to align selected V-ATPase sequences and generate a proteolipid gene phylogeny. Several other alignment programs also supported the resulting Anc3-11 ancestral sequence. In addition, an ancestral Vma16 subunit (Anc16) was reconstructed that
is predicted to have existed at the time of the Anc3-11 proteolipid ring. After testing protein expression of the ancestral genes, complementation tests in *S. cerevisiae* were performed in strains lacking various combinations of proteolipid subunits. The Anc3-11 was able to complement a *vma3Δ* mutant. Surprisingly, this ancestral hybrid was also able to partially complement a *vma3Δ vma11Δ* mutant, converting the yeast 14-subunit V-ATPase into a 13-subunit complex. The low level of complementation results from a defect at the level of V0 assembly; the resulting low level of V-ATPase complex appears to be very active. We are currently generating a heterodimer between Anc16 and Anc3-11 in an attempt to circumvent the assembly defect. We have successfully reconstructed an ancestral V-ATPase proteolipid subunit and shown that it can function within the context of the current-day *S. cerevisiae* V-ATPase complex. <p>[1] Ortlund EA ... Thornton JW, *Science*, 2007

**1503/B661**

**Hph1 and Hph2 Interact with the Sec63 Complex and Are Required for Vph1 Protein Stability in *S. cerevisiae***

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The ER protein Hph1, and its homologue, Hph2, interact with components of the post-translational translocation machinery, and are required for wild type levels of vacuolar acidification. We propose that Hph1 and Hph2 promote the translocation of Vph1, the targeting subunit of the V0 complex of the vacuolar H+-ATPase, into the ER. Hph1 and Hph2 share 23% amino acid identity; each contains a predicted coiled-coil motif and a C-terminal transmembrane domain that anchors the protein to the ER membrane. We performed a membrane-based yeast two-hybrid screen with Hph1 and Hph2 and identified Sec71 and Sec61 as potential interacting proteins. These are two components of the Sec63 complex, which promotes post-translational translocation into the ER. Interaction of Hph1 with Sec71, Sec72, and Sec63 as well as the interaction of Hph2 with Sec71 and Sec63 were confirmed biochemically. Cells lacking Hph1 and Hph2 display growth defects on media containing cell wall damaging agents, oxidative agents, non-fermentable carbon sources and elevated concentration of zinc, cobalt, cesium, and sodium ions. These phenotypes are similar to those of cells with impaired vacuolar H+-ATPase function. In fact, cells lacking Hph proteins display reduced vacuolar acidification, as measured by quinacrine staining, and increased degradation of Vph1. Also, combining *hph1Δ* or *hph2Δ* with mutations that partially disrupt vacuolar H+-ATPase function or assembly, *i.e.* *stv1Δ* or *pkr1Δ*, exacerbates specific growth defects and further compromises vacuolar acidification. These findings suggest that Hph1 and Hph2 act upstream of known vacuolar H+-ATPase assembly factors and may promote the efficient translocation of Vph1 into the ER. Hph1 is a substrate of calcineurin, the Ca2+/calmodulin regulated phosphatase, which is required for yeast survival during environmental stress. An allele of Hph1 that is not dephosphorylated by calcineurin is partially functional, suggesting that calcineurin positively regulates Hph1 activity, and thus efficient production of the vacuolar H+-ATPase, in response to stress.

**1504/B662**

**Hsp70 is Inserted into Artificial Membranes by a Process Mediated by the Presence of Phosphatidylserine.**

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Hsp70 (Hsp72) is the major stress-inducible member of the heat shock protein family. The expression of this protein is crucial in preventing cell death and recovery after different physiological and environmental stresses. Although Hsp70 is primarily located in the cytosol, it has been detected on the cell surface of transformed cells inserted into the plasma membrane. The presence of Hsp70 on the cell surface has been proposed to modulate the immune response in disease conditions. Hsp70 does not contain any hydrophobic domains that could predict its
insertion into membranes. Our objective is to investigate the mechanism of Hsp membrane insertion by incubating pure recombinant Hsp70 with artificial membranes (liposomes). We found significant incorporation of Hsp70 into liposomes composed of phosphatidylserine (PS), which was concentration dependent. In contrast, Hsp90 did not incorporate into PS liposomes. There was no incorporation of Hsp70 into liposomes composed of phosphatidylcholine (PC). Hsp70 inserted into PS liposomes was integrated into the membranes as demonstrated by lack of extraction by sodium carbonate or sonication treatment. Moreover, the protein could only be solubilized by non-ionic detergents. Incorporation of Hsp70 into liposomes was reduced by co-incubation with ADP or ATP (10μM-1mM). These results demonstrate that Hsp70, which does not contain a predictable hydrophobic trans-membrane region, can spontaneously get inserted into a lipid bilayer by a process mediated by the presence of PS and nucleotides. We speculate that the presence of Hsp70 on the cell surface is part of the stress response.

1505/B663

Dual Function of ATP Hydrolysis for Lid Closure and Substrate Release in Group II Chaperonins.

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The primary feature that distinguishes chaperonins is the mechanism employed to close the folding chamber. In the well-characterized group I chaperonin GroEL, the folding chamber is closed via a lid co-factor GroES. Interestingly, group II chaperonins close their folding chamber via ‘built-in’ lids which are composed of the apical protrusions from the rim of the chaperonin cavity. Previously it has been shown that, in the group II chaperonins, ATP hydrolysis triggers the lids to change from the open to closed conformation. but it remains to be seen if, upon closure, the substrate is released inside the chaperonin cavity. Using the chaperonin from the mesophilic archaean bacterium Methanococcus maripaludis (Mm-Cpn), we show that in addition to lid closure, ATP hydrolysis triggers occlusion of the substrate binding sites from the interior of the chaperonin cavity. This occlusion is accompanied by release of the substrate, which is then retained inside the folding chamber by the physical barrier created by the built-in lids. Additionally, we show that lid closure and substrate binding site occlusion are independent events that can be de-coupled. Our data support a model whereby lid closure and substrate release are allosterically coupled, giving rise to productive folding.

Membrane Fusion (1506 - 1513)

1506/B664

Early Membrane Repair by Annexins.

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Repair of the plasma membrane after physical impact is crucial for cell survival and only partially understood. Here we use realtime fluorescence microscopy as well as correlative fluorescence and electron microscopy (EM) to study the contribution of calcium-binding proteins to the initial sealing process. The massive calcium influx after membrane rupture is the largest threat for cell survival. Therefore, a rapid response system is required to limit the intake of calcium ions within 20 - 30 sec. Calcium-sensing proteins with a binding constant above the regular calcium level of a stimulated cell are expected to be instrumental for initiating the early phase of the response process. Good candidates are annexins with their ability to form large two-dimensional arrays by interaction with phospholipid surfaces in a calcium-dependent fashion. We therefore investigated various annexins and found that fluorescently tagged annexin A4 was by far the fastest protein to arrive at the impact site from a blunt needle. Here annexin A4 formed a large aggregate, potentially sufficient to seal the impact site and to prevent further calcium influx. The presence of annexin A4 was also shown by correlative EM and immuno-gold staining of the membranes.
around as well as the vesicles within the impact site. With a delay of 1 - 2 minutes annexins A1 and A6 followed. Annexin A2 with its higher calcium affinity seemed to form protein aggregates in some distance to the impact site, potentially preventing the membrane from tearing. Annexin A5 had little tendency to accumulate at the impact site. By extending translocation experiments to other fluorescently tagged calcium- and lipid-interacting proteins we showed that with a delay of 2 - 3 minutes protein kinase Calpha, its isolated C1 domain but also small G-proteins translocated to the damaged membrane. We conclude that after an initial sealing process involving various annexins an entire signaling machinery is recruited to the impact site, likely to contribute to the next steps in membrane repair.

1507/B665
Structural Basis for Vesicle Tethering by the Dsl1 Complex.
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The delivery of cargo transported within vesicular (or tubular) carriers requires membrane fusion, mediated by SNARE proteins, as well as upstream events that are less well characterized but probably include ‘tethering’, an initial long-range attachment of a vesicle to its target organelle. Among the factors proposed to mediate vesicle tethering are several dimeric coiled-coil proteins, as well as a set of eight or more multisubunit tethering complexes (MTCs). The MTCs contain as many as ten different subunits; the Dsl1 complex, with only three subunits, is the simplest known MTC. To elucidate structural principles underlying MTC function, we have determined the structure of the 250 kDa Dsl1 complex, revealing a tower anchored at its base to two ER SNARE proteins and containing at its tip a flexible lasso for capturing COPI-coated vesicles. Additional conformational flexibility is conferred by several putative hinges within the Dsl1 complex itself. We show that the Dsl1 complex binds to individual SNAREs through their N-terminal regulatory domains, and also to assembled SNARE complexes. Moreover, it is capable of accelerating SNARE complex assembly. Our results suggest that even the simplest MTC may be capable of orchestrating vesicle capture, uncoating, and fusion.

1508/B666
Virus-Induced Gene Silencing to Assign Roles of SNAREs on Tonoplast Protein Trafficking.
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Vesicle shuttling and protein trafficking within the endomembrane system and to the extracellular environment is essential for cell growth and development in plants. In eukaryotic cells, SNAREs (soluble N-ethylmaleimide-sensitive factor protein attachment protein receptor) play roles anchoring other membrane proteins, overcoming the energy required for vesicle membrane fusion. To date, only a handful of plant SNAREs has been characterized, in contrast with their yeast and animal counterparts in the secretory system. The difficulty of studying protein trafficking in plants relies in the fact that knock-out mutations in the secretory pathway result in gametophytic or embryo lethality. Virus-Induced Gene Silencing (VIGS) takes advantage of an RNA-mediated antiviral defense mechanism and can be used to downregulate plant gene expression, without affecting plant viability. In this study, we are proposing to use VIGS as a technology to identify proteins involved in the targeting of membrane transporters to the vacuole. VIGS constructs of SYP21, SYP22, SYP41, SYP61, SYP131, VTI11, VTI12 and AtVPS45, using a vector derived from the DNA geminivirus Cabbage Leaf Curl Virus (CaLCuV), are already synthesized. Plants carrying fluorescent marker proteins fused with the endoplasmic reticulum and the tonoplast or soluble markers will be bombarded at the four-leaf stage with each of the VIGS constructs or the empty vector and analyzed after 2-3 weeks under the confocal microscopy to look for any mis-localization of cargo proteins.

1509/B667
Late Stages of Cell-to-Cell Fusion Initiated by Viral Fusogens Involve Intracellular Curvature-generating Proteins.

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Cell fusion plays an important role in normal physiology (for instance, during fertilization, trophoblast fusion, and myotube formation) and in different pathological conditions (for instance, in viral infections and during carcinogenesis). Much more is known about mechanisms by which specialized fusogenic proteins generate fusion pores than about the mechanisms that control the expansion of these pores. The role of different cellular components in fusion pore enlargement remains to be clarified with different studies implicating different key proteins. To uncouple fusion pore expansion from preceding stages involving membrane docking, expression and activation of fusogens, we have focused on cell fusion initiated by viral fusogens, influenza hemagglutinin and vesicular stomatitis virus G protein. Fusion, in these experimental systems, was triggered by a short-term application of a low pH medium, which allowed us to concentrate on events downstream of the opening of a nanometer-sized fusion pore. We found that, in contrast to local fusion, pore expansion is an active process dependent on metabolic activity of cells and negatively regulated by PKC. Our experiments based on different cell-permeable reagents as well as on microinjection approaches suggest that late stages of syncytium formation are driven by neither microtubule- nor actin- cytoskeleton. Based on a similarity of membrane bending in fusion pore rim and in highly curved membrane compartments such as budding endocytic vesicles we reasoned that fusion pore expansion may involve intracellular curvature-generating proteins. In agreement with this hypothesis, we found that expression of eGFP-tagged GRAF1 and FCHo2 (N-BAR and F-BAR domain proteins, respectively) and microinjection of Epsin ENTH domain promoted syncytium formation. Another curvature generating protein - dynamin - also appears to be involved in late stages of cell fusion, as evidenced by inhibition of syncytium formation by cell-permeable inhibitors of dynamin GTP-ase, dynasore and MitMAB. We propose that expansion of fusion pores involves a local disassembly of actin cytoskeleton and is driven by endogenous curvature-generating proteins gathering at the rim of the pore.

1510/B668
A Single-vesicle Fusion Assay with Tunable Bilayer Interactions Mimics Physiological SNARE Requirements.

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SNARE proteins play a central role in nearly all intracellular fusion reactions: fusion is thought to be driven by formation of trans-SNARE complexes (SNAREpins) through pairing of vesicle-associated v-SNAREs with complementary t-SNAREs on target membranes. The number of SNARE complexes required for fusion is unknown. In past In Vitro experiments SNAREs were reconstituted into bare phospholipid bilayers; these experiments failed to reproduce important physiological characteristics of physiological fusion processes such as the speed of the fusion reaction or the requirement for SNAP25, one of the two neuronal t-SNAREs. Biological membranes, packed with ~30,000 proteins per μm², provide a very different environment for fusion: in order to bring phospholipid bilayers into contact, SNARE proteins In Vivo must presumably perform additional work to clear away this repulsive protein layer. To closely mimic this situation we covered bilayer surfaces with a poly(ethylene glycol) (PEG) brush of ~3-4 nm height using PEG-lipids and we measured single-vesicle docking and fusion events between v-SNARE reconstituted small unilamellar vesicles (v-SUVs) and t-SNARE reconstituted supported bilayers (t-SBLs). Fusion is fast, highly specific, and does require SNAP25. on average, the delay between docking and fusion is ~60 ms. A sharp drop in the overall fusion rate occurs as the number of v-SNAREs per
vesicle is reduced below ~20. Since about half the SNAREs will face the lumen, this implies ~10 SNAREs are required for efficient fusion, which is consistent with the observed distribution of delays between single vesicle docking and fusion events.

1511/B669
The HOPS Complex Stimulates Vacuole Membrane Fusion via Direct Tethering.
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Sec1/Munc18 (SM) proteins are crucial for SNARE-dependent membrane fusion. Mounting evidence suggests that many SM proteins function as part of protein complexes involved in organelle tethering. Yeast vacuole fusion requires the SM protein-containing, heterohexameric HOPS complex; along with the Rab GTPase Ypt7p, 4 vacuolar SNAREs, the SNARE disassembly machinery Sec17p and Sec18p, and specific lipids. We have recently reconstituted subreactions of yeast vacuole fusion using proteoliposomes bearing various combinations of purified membrane components (SNAREs, Ypt7p, lipids) and purified soluble components (HOPS, Sec17p, Sec18p). While our previous studies have shown a central role for HOPS in promoting fusion, its exact mechanism of action is not well understood. Here we show that HOPS directly clusters liposomes that bear one or multiple of its ligands (vacuolar lipids, Ypt7p, SNAREs). By employing poly(ethylene glycol) (PEG) as an artificial liposome clustering agent, we show that tethering of liposomes alone leads to an increase in the rate of SNARE-dependent fusion. However, HOPS yields more fusion than PEG for an equivalent clustering activity, showing that HOPS has functions beyond tethering in promoting fusion. In addition, while Sec17p and Sec18p enhance the ability of HOPS to accelerate liposome fusion, fusion stimulated by PEG is inhibited by Sec17p and Sec18p. The ability of HOPS to act synergistically with the SNARE disassembly machinery, together with previous evidence that HOPS binds multiple SNAREs and proofreads SNARE structure, led us to investigate whether HOPS directly influences SNARE complex assembly. We show here that HOPS does not alter the assembly of SNAREs lacking transmembrane domains or of full-length SNAREs in detergent micelles. In summary, while SNAREs can assist in tethering by acting as ligands for HOPS on membranes, SNAREs are not absolutely required for tethering, since other ligands of HOPS can also support HOPS-mediated clustering of liposomes. Our lack of evidence for the ability of HOPS to directly influence SNARE complex assembly has led us to consider alternative mechanisms for the action of HOPS, and other SM protein-containing complexes, beyond tethering.

1512/B670
Synaptotagmin Mediated Bending of Lipid Membrane is a Critical Step in Calcium Regulated SNARE Fusion.
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It has been proposed that exocytosis involves invagination of the target membrane, involving a localized highly curved site of contact between the bilayers destined to fuse. The vesicle protein synaptotagmin-I binds membranes in response to elevated calcium concentrations, but whether this drives increased curvature of the target membrane to accelerate SNARE mediated fusion has not been determined. Here, we address this question using vesicles with different degrees of curvature. While a tubulation-defective synaptotagmin mutant was able to promote fusion between small, highly curved SNARE-bearing liposomes, it exhibited a marked loss of activity when the membranes were relatively flat. Bending of flat membranes through use of an N-BAR domain rescued the function of the synaptotagmin mutant. We conclude that synaptotagmin induced membrane bending is a critical step in SNARE mediated membrane fusion. Cell. 2009 Aug 21;138(4):709-21.

1513/B671
Minimal Membrane Docking Requirements Revealed by Reconstitution of Rab GTPase-dependent Membrane Fusion from Purified Components.

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Rab GTPases and their effectors mediate docking, the initial contact of intracellular membranes preceding bilayer fusion. However, it is unclear whether Rab proteins and effectors are sufficient for inter-membrane interactions. We have recently reported reconstituted membrane fusion that requires yeast vacuolar SNAREs, lipids, and the homotypic vacuole fusion and protein sorting/Class C vacuole protein sorting (HOPS/Class C Vps) complex, an effector and guanine nucleotide exchange factor for the yeast vacuolar Rab GTPase Ypt7p. We now report reconstitution of lysis-free membrane fusion that requires purified GTP-bound Ypt7p, HOPS complex, vacuolar SNAREs, ATP hydrolysis, and the SNARE disassembly catalysts Sec17p and Sec18p. We use this reconstituted system to show that SNAREs and Sec17p/Sec18p, as well as Ypt7p and the HOPS complex, are required for stable inter-membrane interactions.

Exocytosis (1514 – 1526)

1514/B672
A Protein-Tyrosine Phophatase Phogrin Regulates Autocrine Insulin Signaling Through Direct Interaction with Insulin Receptor.
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Enhanced stimulation of peptide hormone secretion results in endocrine cell growth, and in pancreatic beta-cell, cell growth response to glucose is partly mediated through an autocrine and/or paracrine action of secreted insulin. Phogrin and IA-2, secretory granule-resident protein tyrosine phosphatases, have been shown to be involved in insulin secretion in beta-cell, however, implications at a molecular level are confusing from experiment to experiment. We analyzed biological functions of phogrin in pancreatic beta-cell by an RNA interference technique. Adenovirus-mediated expression of shRNA specific for phogrin efficiently decreased its endogenous expression and resulted in retardation of growth in both cultured beta cell lines and mouse islets. Silencing of phogrin in beta cells abrogated the glucose-mediated mitogenic effect, which was accompanied by a reduction in the level of IRS2 protein, without any changes in insulin secretion and in other protein expressions. Examination of the IRS2 protein stability using the translational inhibitor cycloheximide revealed that phogrin knockdown promoted rapid decline of IRS2 protein in both mouse islets and wild-type beta cells but not in insulin receptor-knockout beta cells. We further found that phogrin directly binds to insulin receptor on the plasma membrane. Their interaction was promoted by the glucose-stimulated insulin secretion that in turn led to stabilization of IRS2 protein. Thus, these observations suggest that phogrin is involved in glucose-promoted beta-cell growth via regulating stability of IRS2 protein by the molecular interaction with insulin receptor. We propose that phogrin and IA-2 function as an essential regulator of autocrine insulin action on pancreatic beta-cell growth.

1515/B673
PICK1 and ICA69 Define Three Maturation Stages of Insulin Granules and Their Deficiency Leads to Impaired Glucose Tolerance.
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Diabetes is a metabolic disorder characterized by hyperglycemia. Insulin, a peptide hormone synthesized and secreted by pancreatic beta cells, is the key regulator of blood glucose. The molecular mechanism governing the budding, trafficking and secretion of insulin is not fully understood. PICK1 (protein interacts with C-kinase 1) is a peripheral membrane protein with both a PDZ (PSD-95/Dlg/ZO1) domain and a BAR (Bin/Amphiphysin/Rvs) domain. ICA69 (Islet Cell Autoantigen 69 kD), an autoantigen initially identified from type-1 diabetes patients, is also a BAR domain protein-containing protein. BAR domains are banana-shaped dimers that bind to lipid
membranes and initiate vesicle formation by sensing membrane curvature or actively bending membranes. We found that PICK1 and ICA69 form heteromeric BAR domain complex in pancreatic beta cells and regulate insulin trafficking. Based on their association with PICK1 and ICA69, insulin granules can be classified into three maturation stages. Furthermore, deficiencies of PICK1 and ICA69 in mice lead to glucose intolerance and other symptoms relevant to diabetes.

1516/B674
Regulation of SNAP-25 Partial Proteolysis, and Exocytosis, by Calpain-10.
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Aims: Diabetes results from a breakdown in glucose homeostasis, and this is ultimately characterized by failure of pancreatic β-cells to secrete sufficient insulin through rapid 1st phase exocytosis. Calpain-10 (CAPN10) was identified as a candidate T2D predisposition gene through genome wide linkage and positional cloning (Horikawa et al, 2000). CAPN10 encodes a 672-amino-acid protease, the 54KDa isoform of which we have previously shown to regulate exocytosis of insulin through partial proteolysis of the t-SNARE, SNAP-25 (Marshall et al. 2005). As calpain activity is dependent upon membrane phospholipid composition, we sought to determine whether phosphoinositide remodeling enzymes might regulate calpain-10 activity and function in pancreatic β-cells. Methods: Knock-down was accomplished using RNAi nucleofection technology (Amaxa, Germany) and viral transfection. INS-1 cells were then incubated +/- secretagogue cocktail, or KCl, and insulin secretion quantified through ELISA (Mercodia, Sweden). Cell lysates, or subcellular fractions, were immunoblotted against calpain-10, SNAP-25, and PI3K. Results and Conclusion: Calpain-10 was found to be concentrated within membrane lipid rafts, along with the t-SNAREs, SNAP-25 and syntaxin-1. Calpain-10 knock-down inhibited both partial proteolysis of SNAP-25 and secretagogue-stimulated insulin secretion. In addition, evidence supports a role for a specific PI3K isofOrm in this process. In summary our data shows for the first time, direct evidence of calpain-10-mediated SNAP-25 partial proteolysis and exocytosis. Reference: Horikawa, Y., N. Oda, N. J. Cox, X. Li, M. Orho-Melander, M. Hara, et al (2000). "Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus." Nat Genet 26: 163-75. Marshall, C., G. A. Hitman, C. J. Partridge, A. Clark, H. Ma, T. R. Shearer and M. D. Turner (2005). "Evidence that an isoform of calpain-10 is a regulator of exocytosis in pancreatic beta-cells". Mol. Endocrinol. 19: 213-24.

1517/B675
Two Pathways for Regulated Dense-core Vesicle Exocytosis in PC12 Cells: CAPS-dependent and CAPS-independent.
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Secretion of hormones and neurotransmitters is a tightly regulated pathway in multicellular organisms that requires Ca2+. Calcium-dependent Activator Protein for Secretion (CAPS) was identified as a factor that primes Ca2+-dependent vesicle exocytosis in permeable neuroendocrine cells. In the current work, the role of CAPS-1 was assessed in PC12 cells by shRNA-mediated knockdown of CAPS-1 to less than 5% of wild-type levels. TIRF microscopy was used to examine dense-core vesicle exocytosis. Depolarization of cells in 56mM K+/2mM Ca2+ (Mild stimulation conditions; MS), led to increases in cytoplasmic Ca2+ and a robust increase in exocytic events. CAPS down-regulated cells exhibited only 20% of the exocytic events under MS conditions compared to wild-type. Stronger stimulation conditions (SS; 95mM K+/2mM Ca2+), in wild-type cells led to even greater increases in cytoplasmic Ca2+ and membrane DAG, but did not further increase the number of exocytic events compared to MS conditions. However, CAPS down-regulated cells exhibited ~80% of their regulated secretory activity under SS conditions. Thus, increased Ca2+ influx shifted exocytosis from mainly CAPS-
dependent to mainly CAPS-independent. To probe the basis of this, we modulated cellular levels of PI(4,5)P2, an essential co-factor for CAPS activity. Increasing hydrolysis of PI(4,5)P2 to DAG reduced exocytic events to 35% of control under MS conditions but had no effect under SS conditions. Overexpression of a 5-phosphatase strongly reduced exocytosis under both MS and SS conditions. These results suggest that a PLC-derived metabolite of PI(4,5)P2 is required for the CAPS-independent pathway. We conclude that there are at least two pathways that regulate dense-core vesicle exocytosis: one that relies on CAPS, PI(4,5)P2, and calcium and a second that is CAPS-independent, requires higher calcium and utilizes a metabolite of PI(4,5)P2 such as DAG. Current efforts are directed at determining whether the second pathway employs Munc13 or PKC.

**1518/B676**

**CAPS Acts as a Tetramer to Promote Regulated Dense-Core Vesicle Exocytosis in PC12 Cells.**

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Multi-cellular organisms utilize the process of regulated exocytosis to secrete neurotransmitters and hormones into the extracellular space. CAPS (Ca2+ dependent Activator Protein for Secretion) is a cytosolic and peripheral membrane bound protein that was discovered for its ability to facilitate regulated exocytosis in permeabilized neuroendocrine cells. Although CAPS was originally purified as a dimer of ~300kda, the significance of its oligomeric state has not been determined. We sought to characterize the native state of CAPS and to determine how oligomerization of CAPS regulates its function. Using native PAGE (polyacrylamide gel electrophoresis) we confirm that CAPS exists primarily as a dimer (94%), however a small fraction also exists as a tetramer (6%). Furthermore, we find that the tetramer is the active form of CAPS and represents the major species of CAPS bound to the plasma membrane of permeable PC12 cells, where it acts to promote regulated exocytosis. Point mutations in the conserved C2 domain affect the oligomerization of CAPS and impair its ability to facilitate regulated exocytosis in permeable neuroendocrine cells. Deletion of the C2 domain results in a 75% decrease in the amount of tetramer and completely abolishes CAPS activity without affecting dimer formation. We conclude that CAPS acts as a tetramer to promote regulated exocytosis and that the C2 domain of CAPS is required for optimal tetramerization.

**1519/B677**

**Exocytotic Events Quantitative Characterization in Total Internal Reflection Microscopy.**

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**INTRODUCTION**

Total internal reflection fluorescence (TIRF) microscopy is a powerful tool for detecting vesicle movement and trafficking in live cells, and has provided significant advances in understanding the molecular mechanisms of regulated hormone secretion in many types of secretory cells. It is now possible to examine the exocytosis-related proteins and secretory vesicle dynamics in real time down to a single exocytosing vesicle and quantifying and classifying them according to such kinetic dynamics. APPROACH to facilitate efficient quantitative characterization of exocytotic events, we extended our automatic subcellular object tracking and characterization technologies called “soft tracking” to 1) track the plasma membrane-docked vesicles; 2) detect exocytotic events; 3) measure track length, track movement kinetics, and fluorescence intensity changes; 4) using track measurement to classify exocytotic response into three types: “residents”, “visitors”, or “passengers”. STUDIES The objective of this study is to validate the performance of the technologies using TIRF microscopy capturing exocytotic responses and docking step of dense-core vesicles of NPY-Venus expressing live PC12 cell movies. for each movie, the quantitative characterization method includes the measurements of 1) Number of exocytotic events, 2) Number of type of exocytotic response, and 3) Real time analysis of number of the plasma membrane-docked vesicles. Our hypothesis is that our methods
achieve similar performance to the best manual method. The manual tracks and exocytotic event truth were created independently. We test the hypothesis using event detection/classification accuracy and counting accuracy metrics. RESULTS The results show good agreement (>90%) of the exocytotic event detection and the exocytotic type classification as well as the exocytotic events counting with the truth. CONCLUSIONS We conclude that the hypothesis is supported with statistical significance. We believe the technologies could standardize quantitative characterization of exocytotic events, and are working to validate them on additional live cell exocytosis movies.

1520/B678
Granule Exocytosis in the Salivary Glands of Live Mice Occurs via Acto-myosin Associated Fusion Events not Involving Compound Exocytosis.
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We have developed an experimental system that enables to image and study exocytosis in live animals by using intra-vital microscopy. As a model we used live transgenic mice expressing GFP under the control of a β-actin promoter, focusing on the agonist-stimulated exocytosis of the secretory granules in the submandibular and parotid salivary glands. In all the major exocrine glands the secretory granules appeared as dark vesicles with a diameter of 0.5-1.5 µm surrounded by soluble GFP. We showed that upon stimulation with agonists of the β-2 adrenergic receptors the granules increase their motility, dock at the apical pole of the acinar cells, where they fuse, releasing their cargo into the acinar canalliculi. Remarkably, we found that secretion occurs via the complete collapse of the granules without kiss-and-run or compound exocytosis and that the excess of membranes is retrieved via compensatory endocytosis. Furthermore, the fate of single granules was followed at a high resolution showing that prior to fusion, actin is recruited onto the granules together with Myosin IIb, an actin dependent motor protein that is expressed in the acinar cells of the submandibular glands potentially regulating the docking step.

1521/B679
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Little is known about how, once a cell fate is specified, transcription factors in differentiating cells regulate the reorganization of cellular structure necessary to perform their specialized physiological functions. Our lab has reported that MIST1, an evolutionarily conserved bHLH transcription factor, is required for the formation of large, specialized secretory vesicles in gastric zymogenic (chief) cells (ZCs) as they differentiate from their mucous neck cell progenitors. Here, we show that MIST1 directly activates transcription of RAB26, a small GTPase that has not been extensively studied but is thought to be expressed only in a limited number of acinar, exocrine cells like the ZC. We show, by in situ hybridization and by qRT-PCR from laser-capture microdissected ZCs from wildtype and Mist1-/- mice, that RAB26 expression is ZC-specific and MIST1-dependent. Optical sectioning and timelapse videomicroscopy showed that eGFP-tagged RAB26 localizes to a post-Golgi cellular compartment, overlapping that of the cation-independent M6PR, indicating that RAB26 is expressed on late endosomes as well as on immature secretory granules budding from the trans-Golgi. Immunostaining for CI-M6PR in tissue showed that the apical, post-Golgi vesicular compartment is markedly stunted in Mist1-/- ZCs, suggesting a mechanism wherein MIST1 is required for formation of large secretory granules, because it acts through RAB26 to expand the cellular compartment where those zymogen granules mature. To test this hypothesis, we developed gastric cell lines stably expressing MIST1 and transfected them with RFP-tagged pepsinogen C, a key secretory product of ZCs. Those cells formed large secretory granules that depended on RAB26 because co-transfection with a dominant negative RAB26 plasmid abrogated their formation. Taken together, our data establish for the first time the
molecular process by which a transcription factor directly induces fundamental cellular architecture changes by increasing transcription of a specific cellular effector that acts to organize a unique subcellular compartment.

1522/B680

RAB27a and RAB27b Regulate the Exocytosis of Neutrophil Granules by Independent Mechanisms.

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Neutrophils rely on exocytosis to mobilize receptors and adhesion molecules and to release microbicidal factors. This process must be strictly regulated because uncontrolled release of toxic proteins would be injurious to the host. In Vivo studies showed that the small GTPase Rab27a regulates azurophilic granule exocytosis. Using mouse neutrophils deficient in Rab27a (ashen), Rab27b (Rab27b knockout) or both (Rab27a B double KO), we investigated the role of the Rab27 isoforms in neutrophils. We found that both Rab27a and Rab27b deficiencies impaired azurophilic granule exocytosis. Rab27a KO neutrophils showed upregulation of Rab27b expression which did not compensate for the secretory defects observed in Rab27a-deficient cells suggesting that Rab27 isoforms play independent roles in neutrophil exocytosis. The expression of Rab27a in Rab27b KO cells is similar to its expression in wild type neutrophils. Total internal reflection fluorescence microscopy analysis showed that Rab27a-deficient (ashen) neutrophils have a decreased number of azurophilic granules near the plasma membrane. The number of granules in proximity to the plasma membrane was also decreased in Rab27aB double knockout neutrophils. Rab27a KO but not Rab27b KO neutrophils, failed to mobilize cytochrome b558 to the plasma membrane although mobilization to the phagosome was not affected. The defect was associated with an impaired oxidative response to soluble stimuli but normal phagosomal oxidative response in Rab27a-deficient neutrophils. Exocytosis of secretory vesicles in Rab27-deficient neutrophils was functional, suggesting that Rab27 GTPases selectively control the exocytosis of neutrophil granules.

1523/B681

Phosphatidylinositol 4-phosphate Facilitates Recruitment of the Rab GEF Sec2p.

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Sec2p is the guanine nucleotide exchange factor (GEF) that activates the Rab GTPase Sec4p on secretory vesicles. Sec2p binds the GTP-bound form of Ypt32p, a Rab that works upstream of Sec4p. Thus Sec2p is a GEF for one Rab and an effector for another Rab. This mechanism is called a Rab-GEF cascade and is important for the spatial regulation of Rab activation. Sec2p also binds Sec15p which is a component of the Exocyst complex required for vesicle tethering to the plasma membrane. Since Sec15p is an effector of Sec4p, this interaction potentially generates a positive feedback loop on secretory vesicles. Ypt32p and Sec15p compete for binding to Sec2p in vitro, raising the question of how these two mechanisms are coordinated in vivo. We show here that Sec2p binds phosphatidylinositol 4-phosphate (PI4P) and that the PI4P generated by the Golgi-associated PI4 kinase, Pik1p, is needed together with Ypt32p-GTP for Sec2p recruitment. Recombinant Sec2p binds to PI4P-containing liposome via its positive charge clusters. In wildtype yeast cells, Sec2 localizes to the secretory vesicles concentrated at the bud tip and mother-daughter neck. However, in a pik1 mutant Sec2p is mislocalized to the cytoplasm. In vitro, PI4P liposomes inhibit the Sec2p-Sec15p interaction, but not the interaction of Ypt32p-GTP with Sec2p. Co-immunoprecipitation studies showed that the Sec2p-Sec15p interaction is enhanced in a pik1 mutant. Therefore, by locally blocking Sec15p binding, PI4P may promote the recruitment of Sec2p by Ypt32p. The level of PI4P appears to be reduced once vesicles reach...
secretory sites, allowing Sec15p to replace Ypt32p on Sec2p as vesicles mature. We also show here that sec2 mutants selected for reduced Ypt32p-binding fail to localize, leading to secretory and growth defects. We found a striking inverse correlation between Ypt32p-binding and Sec15p-binding: mutants defective for binding Ypt32p showed enhanced binding to Sec15p. Those sec2 mutants also showed an altered limited proteolysis pattern. We propose that Sec2p has two conformational states, one favoring Ypt32p binding and another favoring Sec15p binding. PI4P may specify the binding partner for Sec2p.

1524/B682
Rab8a Recruits the Exocyst to the Discharging Contractile Vacuole in Dictyostelium. M. Essid, T. Soldati; Biochemistry, University of Geneva, Geneva, Switzerland

The exocyst is an octameric tethering complex functioning in cellular processes such as focal exocytosis, cytokinesis and cell migration. In Dictyostelium we identified a single ortholog for each subunit. The exocyst transiently localised at sites of post-lysosome exocytosis, but the most robust localisation was at the contractile vacuole (CV) during discharge. The CV regulates the osmotic equilibrium by repeated cycles of water pumping and expulsion. We hypothesize that the exocyst functions in tethering the CV to the plasma membrane (PM) prior to SNARE interaction and pore formation. Therefore, we monitored the exocyst in mutants impaired in CV function. Drainin, a Rab-GAP-like protein, is proposed to be necessary for pore formation, as drainin-null cells show a strongly enlarged CV. Block of discharge led to enrichment of GFP-Sec15 at the contact site between CV and PM, which we interpret as over-tethering. LvsA is a BEACH-domain protein of the Shediak Higashi family. LvsA-null cells showed enrichment of GFP-Sec15 and Rab8a at the site of discharge. We speculate that LvsA functions in “de-tethering” of the CV from the PM before a new cycle of water pumping. Yeast 2-Hybrid screens and co-immunoprecipitations with exocyst subunits identified interaction partners and potential regulators of the complex, including several small GTPases and their regulators. Among these, Rab8a localised to the CV shortly before and during discharge, and interacted with the Sec15 in both its GDP and GTP-locked forms. Regulated expression of these Rab8a mutants had a strong impact on CV size and morphology. Rab8a CA was present on the CV, but its expression resulted in CV fragmentation to numerous small vacuoles found clustered in a crescent at the PM. In contrast, Rab8a DN-expressing cells showed inflated CV impaired in their discharge. Interestingly, Rab8a DN did not localise to the CV but was found diffusely in the cytosol, but still associated with Sec15. This strongly suggests a working model in which Rab8a recruits Sec15 and the exocyst to the CV in a GTP-dependent manner and regulates tethering of the CV to the PM. We propose that the Rab8a/exocyst complex controls the discharge cycle downstream of Drainin, upstream of LvsA.

1525/B683
An Internal Domain of Exo70p is Required for Actin-independent Localization and Mediates Assembly of Specific Exocyst Components.
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The exocyst is an octameric protein complex required for tethering secretory vesicles to the plasma membrane in preparation for fusion. Structural evidence suggests that each subunit is rod-shaped and they align in a side-by-side fashion to perform their tethering function. In this study, we demonstrate that three of the four domains of the exocyst subunit Exo70p are essential. An internal domain C is dispensable for growth but is required for the actin-independent localization of Exo70p. Domain C mediates an interaction with Rho3p and a deletion of it (exo70ΔdC) is synthetically lethal with mutations in genes encoding other exocyst subunits and essential components of the secretory machinery and causes partially defective exocyst assembly. The exocyst subunit Sec3p also contains a Rho interaction domain that is required for its actin-independent localization and if deleted (sec3ΔM), it is synthetically lethal with exo70ΔdC, even in the presence of high copy number suppressors that bypass complete deletions of either
single gene. Loss of the Exo70p-Rho3p interaction does not account for the synthetic lethality interactions or the defective exocyst assembly. The results suggest that either Exo70p or Sec3p must associate with the plasma membrane in order for the exocyst to function as a tether.

1526/B684
The Sterol-Binding Protein Kes1/Osh4p Regulates Polarized Vesicular Transport.
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The yeast Oxysterol-binding protein homologues (OSH) are a seven-member gene family that is implicated in the non-vesicular transport of sterols between membranes. To the contrary, we found that Kes1/Osh4p directly affects vesicular transport by facilitating the exocytosis of post-Golgi vesicles to sites of polarized cell growth. Previously, using a mutant conditional for OSH function, we found that exocytic vesicles fail to dock at the plasma membrane and accumulate in the daughter bud, resulting in the accumulation of the normally exocytosed cargo Bgl2p. To elucidate the role of Osh proteins in vesicular transport we used time-lapse three-dimensional (4D) spinning disc confocal microscopy. We found that Osh4p co-localized with Sec4p marked exocytic vesicles as they moved from mother cells to the plasma membrane of their daughter buds. Fittingly, Osh4p co-fractionated with purified Bgl2p containing vesicles. Overall, this data suggests that Osh4p directly facilitates the docking of exocytic vesicles at sites of polarized growth. To elucidate the mechanism of Osh4p in vesicle docking, we assayed for Osh protein interactions with the regulators of vesicle docking in vivo. Consistent with our previously published genetic interactions, we found that Osh4p formed complexes with the small GTPases Cdc42p, Rho1p, and Sec4p, and the exocyst subunit Sec6p. Furthermore, in a pathway that regulates CDC42-dependent polarized cell growth, we found that the myosin-interacting kinesin SMY1 represses OSH4. Although Osh4p directly affected polarized exocytosis, its role in sterol trafficking was unclear. Contrary to what is predicted for a sterol transporter, the inability of the Osh4(Y97F) mutant protein to bind sterols did not cause its inactivation. Rather, Osh4(Y97F)p was hyperactivated and dominant lethal. Yeast cells with reduced expression of Osh4(Y97F)p were hypersensitive to SEC4 dosage and have depolarized Sec4p suggesting that the lethality is linked to cell polarity defects. Thus, we propose that sterols are signaling ligands for Osh4p regulation of vesicle docking, and Osh4p has an independent role in sterol trafficking but not as a transporter.

Trafficking in Polarized Cells (1527 – 1536)

1527/B685
Role of Varp, a Rab21 Exchange Factor and TI-VAMP/VAMP7 Partner, in Neurite Growth.
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The vesicular SNARE TI-VAMP/VAMP7 was previously shown to mediate an exocytic pathway involved in neurite growth but its regulation is still largely unknown. Here we show that TI-VAMP interacts with the Vps9 domain and ankyrin repeat containing protein (Varp), a Guanine nucleotide exchange factor (GEF) of the small GTPase Rab21, through a specific domain herein called ID. Varp, TI-VAMP and Rab21 colocalize in the perinuclear region of differentiating hippocampal neurons and transiently in transport vesicles in the shaft of neurites. Silencing the expression of Varp by RNAi or expressing ID or a form of Varp deprived of its Vps9 domain impair neurite growth. Furthermore the mutant of Rab21 defective in GTP hydrolysis enhances
neurite growth. We conclude that Varp is a positive regulator of neurite growth through both its GEF activity and its interaction with Ti-VAMP.

1528/B686
Mechanisms of Myelin Membrane Growth in a Cellular Model System.
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The history of glial research spans more than a century. Nevertheless, formation of myelin has remained one of the big questions of cell biology. Myelin is an insulating multilayer of plasma membrane, which glial cells termed oligodendrocytes wrap around axons. As visualization of membrane trafficking in these tightly packed, nanometer scaled layers is an extremely challenging task, we have established a 2D model of myelin sheath. This model enabled us to investigate how myelin grows, where intracellular transport of myelin proteins takes place and where newly made myelin membrane is added to the expanding myelin sheet. Cultured primary mouse oligodendrocytes (pOLs) produce flat membrane extensions, which strikingly recapitulate the biochemical and morphological microcomposition of myelin in vivo. Surprisingly, these domains develop their identity even in cell culture without requirement for wrapping around axons. We have characterized these compact and non-compact myelin-like domains of the pOLs plasma membrane using immunofluorescence and subcellular fractionation in conjunction with western blotting techniques. We showed that the more compact areas ("sheets") are enriched in myelin basic protein (MBP) and these membranes are lighter in biochemical tests, while less compact ("processes") domains are enriched in 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) and myelin-associated glycoprotein (MAG) and proved to be heavier in sucrose gradients. We found vesicular transport and fusion machineries (cytoskeleton, exocyst, SNAREs) to be localized in the non-compact regions of the cells. Total internal reflection fluorescence microscopy (TIRF) allowed us to pinpoint fusion events of a compact myelin protein tagged with a pH-sensitive GFP variant. Using metabolic labeling, we could detect time-induced shifts of S35-containing newly synthesized proteins from the heavier to the lighter membrane fractions. These data suggests novel directionality for myelin membrane growth: From the processes into the sheet domains. We are currently confirming these findings with morphological pulse-chase and fluorescence recovery after photobleaching (FRAP) approaches.

1529/B687
Quantum Dot Labelled IGF-I: A Novel Mechanism to Study IGF-signal Transduction Across an Epithelial Bilayer.
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During pregnancy, transfer of nutrients between mother and fetus occurs at the epithelial layer of the placenta (syncytiotrophoblasts; ST). The ST is non-proliferative and its expansion is maintained by the growth, differentiation and fusion of underlying (basal) progenitor cells known as cytotrophoblasts (CT). Application of insulin-like growth factors (IGFs) to explants of human villous placental tissue induces CT proliferation. A trans-synctial pathway for signal transmission is therefore required, but its mechanism has not been established. IGF1R is located both on the ST surface and on CT. We have evidence of a signalling pathway in CT that is activated by IGF: the phosphatase SHP-2 is present in CT but not ST, and siRNA knockdown shows that it is required for IGF-induced CT proliferation. Furthermore, after removal of ST, IGF can directly influence CT proliferation. We hypothesise that IGF can cross the outer epithelial (syncytial) layer to gain access to receptors in CT. To test this we have developed methodology to track exogenous IGF in tissue explants. Biotinylated IGF-I (50nM) was loaded onto streptavidin-conjugated Quantum Dots (QDs) and activity (western blotting to show stimulation of IGF1R phosphorylation) of the resulting complex was confirmed in mouse fibroblasts overexpressing IGF1R. QD-IGF-I was delivered to first trimester human placental tissue and tracked using real time fluorescence microscopy for up to 4 days. Location of QD-IGF-I changed with time: 30 minutes after initial exposure, it was present on
the ST surface, by 1 hour it was visible within ST and at 6 hours, dots were distributed throughout the ST and on the surface of underlying CT. Immunohistochemical analysis revealed that QD-IGF-I enhanced both CT proliferation (Ki67) and survival (M30) (P<0.05; n=4). These data demonstrate trans-epithelial transport of a key growth factor prior to activation of a signalling pathway in underlying cells. Since IGF was delivered from the maternal side of the placenta, the identification of this pathway offers a new insight into the control of placental (and hence fetal) growth from the maternal environment.

**1530/B688**

**Increased Antibody Content in Tears of plgR Knockout Mice.**

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The polymeric Ig receptor (plgR) mediates basal to apical transcytosis of dimeric IgA (dIgA) or pentameric IgM across epithelial cells, including lacrimal gland acinar cells, which are responsible for the regulated secretion of tear proteins into tear fluid. When trafficked to the apical surface, the extracellular domain of the plgR with bound dIgA is proteolytically cleaved into secretory IgA (sIgA) and is released into tear fluid. The antibody content of the tears from plgR knockout mice was compared with wild-type C57BL/6 to evaluate possible compensatory responses which might maintain tear antibody composition in response to deficiencies in IgA/IgM transport. Tears were collected from 6 and 12 week old wild-type and plgR knockout mice. Western blots with antibodies directed against IgA, IgM, and IgG were performed. Tears from both 6 and 12 week old plgR knockout mice interestingly had a greater abundance of IgA, IgM, and IgG than did the wild-type, as shown by Western blots. The tears of the 12 week old plgR knockout mice had a slightly higher antibody content than tears of 6 week old mice. To determine whether another Ig receptor, the FcRn receptor, may be involved in the transcytosis of these immunoglobulins, the expression of the FcRn receptor was tested through reverse transcriptase real time PCR of lacrimal gland mRNA and Western blots with an anti-FcRn antibody. Gene expression was found to be similar in wild-type and plgR knockout mice. Western blots of lacrimal gland lysate also revealed similar FcRn protein levels between wild-type and plgR knockout mice. The increased antibody content in the tears of plgR knockout mice may indicate the presence of a compensatory pathway for immunoglobulins to reach the tears, although overexpression of the FcRn receptor does not appear to be the mechanism for increased antibody secretion. *(This study was supported by NIH grant EY016985)*

**1531/B689**

**Live Cell Imaging of GFP-Rab11a in Lacrimal Gland Acinar Cells Reveals Distinct Vesicle Pools.**

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Objective: to determine the role of rab11a in membrane trafficking in lacrimal gland acinar cells (LGACs), specialized epithelial cells that secrete tear proteins apically in response to secretagogues. Methods: Primary cultured rabbit LGACs were co-transduced with Adenovirus (Ad) constructs encoding Rab11a tagged with Green Fluorescence Protein (GFP) on the N-terminal, in parallel with Ad encoding the Tet-on transactivator. GFP-Rab11a expression was induced with 10μg/ml doxycycline. LGACs were treated with or without the secretagogue, carbachol (100μM, 1h), and/or nocodazole (33μM, 2h). The distribution of GFP-Rab11a was analyzed using confocal fluorescence microscopy while membranes from transduced cell lysate were fractionated using sorbitol density gradient centrifugation and analyzed by Western blotting. Results: GFP-Rab11a-enriched vesicles are mostly localized beneath the apical membrane and with the subapical microtubule (MT) network. Live cell imaging revealed rapid, directed movement of small GFP-Rab11a-enriched apical vesicles as well as tubules between apical and basolateral membranes, but also showed large (>0.5μm) and distinct subapical GFP-Rab11a-enriched
vesicular structures that remained static. Nocodazole redistributed apical GFP-Rab11a-enriched vesicles to the basolateral domain. Fluorescent dextran endocytosed into LGAC was not significantly enriched in GFP-Rab11a-enriched vesicles either without or with carbachol. Membrane fractionation showed enrichment of both GFP-Rab11a and EEA1 on intermediate density membranes, but their redistributions observed in response to nocodazole or carbachol were distinct. Although partially co-localized with Rab3D and Rab27b-enriched secretory vesicles, most GFP-Rab11a was not detected on secretory vesicles and appeared to label a spatially distinct vesicle population. Conclusion: Rab11a-enriched vesicles participate in trafficking between apical and basolateral membranes along the MT network. This vesicle pool is not highly enriched in fluid phase markers and may represent transport vesicles carrying specific cargo from endosomal pools to subapical secretory pools including those marked by mature secretory vesicle markers.

1532/B690
Shuttling of Galectin-3 between Lipid Raft-dependent Endocytic and Raft-independent Exocytic Traffic.
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In epithelial cells, the beta-galactoside binding lectin galectin-3 mediates the non-raft dependent glycoprotein targeting to the apical membrane domain. In this study, we aimed to identify the non-classical intracellular trafficking of galectin-3. By studying fluorescent fusion proteins in living cells, we could show that galectin-3 accumulates intracellularly in acidified endosomes before being exocytosed at the plasma membrane. TIRF-m studies of the apical surface of polarized MDCK cells revealed that galectin-3 is enriched in tubular and vesicular Rab11-positive recycling endosomes in the vicinity of the apical cell surface. Recycling of the lectin into the cell interior is facilitated by non-clathrin mediated endocytosis. This uptake is lactose-inhibitable and requires carbohydrate binding of galectin-3. Moreover, endocytosis of galectin-3 comprises the association with lipid rafts, while exocytosis of the lectin does not employ alliance with these membrane microdomains. Our findings demonstrate that galectin-3 constantly cycles between endosomes and the apical membrane of epithelial cells thereby alternating between lipid-raft and non-raft transport platforms.

1533/B691
Protein Sorting in the Direct and Indirect Apical Targeting Pathways: Simultaneous Sorting of Apical and Basolateral Cargo In Transcytotic Pathway.
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Epithelial cells employ two strategies for apical/luminal protein transport in the secretory pathway. Columnar-epithelial cells, such as those of the kidney, utilize predominantly the direct route where proteins are transported from the trans-Golgi network (TGN) to the apical membrane. By contrast, single-spanning membrane proteins in hepatic cells are instead targeted from the TGN to the basolateral surface and delivered to the luminal domain (bile canaliculus) upon endocytosis in a process referred to as transcytosis (i.e. indirect targeting). Here we have visualized the sorting and trafficking of apical (human dipeptidyl peptidase IV) and basolateral (vesicular stomatitis virus glycoprotein) cargo in living cells using multicolour imaging of green fluorescent protein variants. We observed a predominant simultaneous TGN sorting of apical and basolateral cargo in common tubulo-vesicular structures in primary cultures of hepatocytes and hepatic WIF-B9 cells while in columnlar, kidney-derived MDCK cells both proteins are segregated into independent transport carriers. These findings suggest that the direct and indirect apical pathways differ in their ability to segregate apical from basolateral proteins at the level of the TGN.

1534/B692
Multiple Biosynthetic Trafficking Routes for Apically Secreted Proteins in MDCK Cells.
Newly synthesized apical and basolateral membrane proteins can traverse endocytic intermediates en route to the plasma membrane; however, the route secreted proteins take to the surface has not been clearly elucidated. A priori, it is not obvious why cells might sort apically secreted proteins via endocytic compartments, as endocytosed soluble proteins are efficiently shunted to lysosomes or transcytosed to the basolateral surface. However, some newly synthesized soluble proteins may associate with membrane receptors during surface delivery. Here, we examined the route of two soluble proteins that are delivered apically via distinct mechanisms in polarized Madin Darby canine kidney (MDCK) cells. Efficient apical sorting of a glycosylated version of mouse growth hormone (gmGH) requires its N-glycans, whereas apical secretion of a truncated mutant of endolyn (Ensol) is glycosylation-independent. To test the possible involvement of apical early endosomes in secretion of these proteins, we internalized horseradish peroxidase-conjugated wheat germ agglutinin (HRP-WGA) from the apical surface and functionally inactivated this compartment by subsequent incubation with diaminobenzidine and H2O2. Neither gmGH nor Ensol secretion was disrupted under these conditions, suggesting these proteins do not traffic through apical early endosomes. However, apical secretion of gmGH, but not Ensol, was partially inhibited in a cell line stably expressing a dominant-negative mutant of myosin Vb that disrupts exit from apical recycling endosomes (ARE). Because galectin-3 has been implicated in biosynthetic apical sorting of some glycosylated membrane proteins, we asked whether this lectin is also involved in gmGH trafficking. SiRNA mediated knockdown of galectin-3 had no effect on the rate or polarity of gmGH secretion, suggesting that a distinct mechanism is responsible for the efficient apical delivery of this protein via the ARE. Our results demonstrate that two soluble proteins with different apical targeting signals take distinct routes to the apical surface.

1535/B693
Regulation of Endosomal Clathrin and Retromer Mediated Endosome to Golgi Retrograde Transport by the J-domain Protein RME-8.
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After endocytosis most cargo enters the pleiomorphic early endosomes where sorting occurs. As endosomes mature transmembrane cargo can be sequestered into inwardly budding vesicles for degradation, or can exit the endosome in membrane tubules for recycling to the plasma membrane, the recycling endosome, or the Golgi apparatus. Endosome to Golgi transport requires the retromer complex. Without retromer, recycling cargo such as the MIG-14/Wntless protein aberrantly enters the degradative pathway and is depleted from the Golgi. Endosome associated clathrin is also affects the recycling of retrograde cargo and has been shown to function in the formation of endosomal subdomains. Here we find that the C. elegans endosomal J-domain protein RME-8 associates with the retromer component SNX-1. Loss of SNX-1, RME-8, or the clathrin chaperone Hsc70/HSP-1 leads to over-accumulation of endosomal clathrin, reduced clathrin dynamics, and missorting of MIG-14 to the lysosome. Our results indicate a mechanism whereby retromer can regulate endosomal clathrin dynamics through RME-8 and Hsc70, promoting the sorting of recycling cargo into the retrograde pathway.

1536/B694
Endocytic Trafficking Regulates C. elegans PAR-Protein Dependent Embryonic Polarization and Asymmetric Division.
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We are interested in understanding asymmetric cell division, a process that relies on the polarization of the cell prior to its division and allows the generation of cell diversity. In the *C. elegans* zygote, polarity is established by actomyosin-dependent contractility events along an antero-posterior axis and leads to the formation of two unequal cells with distinct fates. The evolutionary conserved PAR proteins are essential for these events and are separated in two exclusive cortical groups: the PAR-3/PAR-6/PKC-3 complex at the anterior cortex and PAR-1 and PAR-2 at the posterior cortex. The molecular mechanisms regulating PAR protein function remain elusive. While directed vesicular trafficking is known to maintain the polarity of epithelial cells, the importance of this process in establishing epithelial cell polarity is not well understood. Here we report that polarization and asymmetric division of the *C. elegans* embryo depends on the endocytic trafficking machinery. We found that disrupting several genes involved in early steps of the endocytic pathway could suppress par-2 lethality suggesting that they function in cell polarity and asymmetric cell division. In addition, disruption of several of these genes resulted in polarity-associated embryonic defects. Interestingly, we observed that PAR-6, in addition to its cortical localization, localizes in cytoplasmic puncta during the establishment of polarity where it colocalizes with PAR-3 and PKC-3. Strikingly, these puncta also colocalize with different vesicular proteins, such as RAB-5, suggesting that PAR proteins navigate in different endocytic compartments. Furthermore, the presence of these endocytic puncta relies on the unconventional class V myosin HUM-2, a known regulator of vesicle trafficking. Taken together, these results indicate that genes regulating vesicular trafficking participate in PAR-protein dependent embryonic polarity in *C. elegans*, and suggest a model in which they function in parallel to actomyosin contractions to localize PAR proteins. Since anterior PAR proteins are themselves positive regulators of endocytosis, our results could reflect the existence of a feed-back loop mechanism involved in the establishment of cell polarity.

**New Emerging Technologies for Cell Biology II (1537 – 1550)**

**1537/B695**

**Microfluidic Array for 3D IrECM Culture of Cancer Cells.**

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There is widespread evidence that culture of cancer cell malignancy and response to drugs in a laminin rich, 3D extracellular matrix (IrECM) is more similar to the In Vivo condition than in traditional 2D monolayer. Most approaches involve manual manipulation of gels, making experiments costly, time consuming, and highly variable. Here, we present a novel microfluidic array suitable for establishing 100 nanoliter chambers of Matrigel with embedded cancer cells. This small volume allows for increased control over gel uniformity, while minimizing reagent usage. The chambers’ height of 100 microns provides for a consistent focal plane for cell microscopy. In addition, an adjacent flow channel enables continuous flow perfusion of culture medium (or exposure solutions) into the chamber to nourish the cells for 3-5 days. This unique microfluidic design overcomes the mass transport problems associated with all other gel-based culture methods. The array is designed to operate on a standard 96 well plate format. Cells and gel are mixed off-chip, and pipetted into the loading well of the microfluidic plate. A surface tension mediated flow transports the cells into the culture chamber, where they accumulate within the polymerizing gel. No additional attachments or equipment are necessary to run the microfluidic plate. Each plate contains 24 independent 3D culture flow units. We demonstrate operation of this device with long term culture of mammary breast epithelial cell lines in Matrigel. Known cytotoxic compounds were exposed to cells in this format and compared to the same device without Matrigel. Standard assays including fluorescence microscopy and plate reader based assays were used to assess cell viability and response to treatment. The key technology innovation presented by this work is an improved 3D IrECM culture method, providing improved...
throughput, reduced reagent usage, better data quality, and reduced cost. The compatibility with standard 96 well plate operation and microscopy methods makes the device amenable to a large number of existing assays. Automated flow experiments simulate In Vivo tumor conditions and may be useful in morphological studies, cancer cell invasion studies, and drug treatment tests.

1538/B696
Cell-Culture in Microfluidic Devices with Controlled Oxygen Levels.
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Levels of oxygen experienced by mammalian cells In Vivo are substantially lower than the oxygen concentration, [O2], found in the atmosphere. The average pO2 in tissue is around 35 mmHg (equivalent to the medium at equilibrium with 4.5% [O2]), and the base level of [O2] needed for cell maintenance is estimated at ~3%. Therefore, cell cultures open to atmosphere are usually at non-physiological high levels of oxygen. We have developed a microfluidic platform that enables generation of stable dissolved oxygen levels, unaffected by ambient [O2] or by cellular oxygen uptake rates. The platform consists of microfluidic devices and a novel computer controlled multi-channel gas mixer generating mixtures of N2 and O2 (plus 5% CO2). Two types of microfluidic devices were constructed and tested to simultaneously expose cells to different graded levels of [O2] or to well-defined spatial gradients of [O2]. The devices of the first type were used to expose Chinese hamster ovary (CHO) cells to 9 different [O2] in overnight experiments to measure CHO cell response to various degrees of hypoxia. The devices of the second type were used to measure 2D-migration of human umbilical vein endothelial cells (HUVECs) on fibronectin coated substrate under several different [O2] gradients. A modified version of the devices made it possible to maintain cells in a 3D extracellular matrix gel with an oxygen gradient imposed across the gel. This modified version was used for measuring migration of cytotrophoblast cells imbedded in Matrigel and for visualizing vascular remodeling of dissociated mouse retinal tissue. Our findings with CHO cells show agreement with the literature, with cell viability quickly dropping from 90% at [O2] = 5% to 10% at [O2] = 3%. Furthermore, cells displayed less cell-to-cell contact and spreading below [O2] = 6%. No detectable bias in the migration of cultured HUVECs was observed over 2 days, suggesting a possible paracrine mechanism in directing cell migration. Lastly, cytotrophoblasts showed alignment along the direction of [O2] gradients over 2 day cultures in Matrigel and 3D-cultures of dissociated retinal tissue cells displayed vessel-like formations at the lower oxygen concentrations over a 5 day culture.

1539/B697
Development of Advanced Cell Culture Surfaces that Improve Cell Attachment and Growth.
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Surface modifications of cell culture vessels significantly affect cell functions that include attachment, growth, and differentiation. Here we evaluated two chemically-defined animal-free advanced cell culture surfaces - BD PureCoat™ amine, a positively charged surface and BD PureCoat™ carboxyl, a negatively charged surface for cell attachment, growth, and differentiation. We observed that both surface treatments support attachment and faster growth of many cell lines such as BHK-21, MRC-5, HEK-293, HT-1080, and HepG2 compared to standard tissue culture (TC) vessels. Notably, cell growth advantages (10-60% vs. TC) were also observed in reduced-serum supplemented and/or serum-free medium. Freeze-thaw recovery and attachment of LnCAP cells on the carboxyl surface was significantly better (p<0.05) than TC plates. We also evaluated growth and differentiation of primary cell cultures as they are more tenuous systems and often require attachment factors to adhere to the surface. We found that the amine surface provides cell attachment and differentiation of primary neuronal cells (rat cerebellar granule, cortical neuronal and astrocytes) whereas these cells do not adhere on uncoated TC vessels. We evaluated growth of primary astrocytes on the amine surface and found increased cell growth (>50%) compared to TC plates. Furthermore, neural cells grown on the amine surface continue to express specialized phenotypic markers such as Glial Fibrillary Acidic Protein and...
tubulin IIIβ characteristic to the cells tested. Both the carboxyl and amine surfaces are defined animal-free advanced cell culture surfaces that provide an alternative to biological coatings without compromising cell attachment and function.

**1540/B698**

**Lentiviral Transduction of Hematopoietic Stem and Progenitor Cells.**

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Objectives: Recently HIV-based lentiviral vectors have emerged as powerful tools for transgene delivery into mammalian stem cells. Lentiviral transduction of hematopoietic stem cells could be used for gene therapy approaches development. Therefore careful analysis of transduced stem cells properties, their proliferation and differentiation potential would be of great importance.

Methods: Lin-c-kit+(LK)-, Lin-c-kit+Sca-1+(LKS)-, or CD105+Sca-1+(CS)-cells were isolated from murine bone marrow (BM) using magnetic separation and incubated with HIV-based lentivector harboring dsRed gene under the control of MSCV promoter. To test the results of transduction lethally irradiated CBA×C57Bl6 mice were administrated with 20000, 1600 or 800 transduced LK-, CS- and LKS-cells respectively for long-term hematopoietic reconstitution. BM cells of reconstituted mice were stained with MAB to CD34, Sca-1, c-Kit and “lineage” markers CD3ε, Gr-1, Ter119, B220 and studied by FACS. Transgene incorporation into genomic DNA was verified by PCR. Results: Transduced LK-, LKS- and CS-cells restored hematopoiesis of lethally irradiated mice. BM cells of 65% chimeras reconstituted with LK-cells (n=21) bore integrated transgene in early (1,5-4 months) and late (8-12 months) periods after irradiation. DsRed expression was observed in BM cells 41% of those mice. BM cells of chimeras, reconstituted with LKS- (n=23) and CS-cells (n=21), 31% and 23%, respectively, contained integrated transgene 8-12 months after irradiation. DsRed expression was observed in BM cells of 15% and 8% of those chimeras. Portions of cells, positive for CD34, Sca-1, c-Kit, CD3ε, Gr-1, Ter119 and B220 in BM cells of chimeras did not differ from the ones in BM of normal mice. Conclusions: LK-, LKS- and CS-BM cells transduced with HIV-based lentivector harboring reporter gene were capable of restoring hematopoiesis in lethally irradiated mice. This vector enables transgene incorporation into short-term and long-term repopulating stem cells. There was no noticeable toxic effect of incorporated lentiviral constructions on stem cell functioning. Transgene was preserved for 12 months in BM cells in vivo. Funding: This research was supported by RFBR grant #07-04-01456.

**1541/B699**

**Combined Biopreservation Solution and Gel Component Improves Recovery of Preserved Cells.**

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Background: Preservation and transport of biologics has traditionally been achieved through suspension in a liquid medium. The liquid environment does not provide physical support for the biologic during transport and subsequent preservation and the biologics are therefore exposed to physical stresses in addition to biochemical stresses associated with biopreservation such as apoptosis and necrosis. Methods: Normal Human Dermal Fibroblasts (NHDF) and Human Mesenchymal Stem Cells (HMSC; Lonza) and human sperm cells were cultured following standard methods. Gel solutions were prepared by combining gelatin with either cell culture media, or HypoThermosol® FRS (HTS-FRS; BioLife Solutions), or CryoStor™ (CS5; BioLife Solutions). for cryopreservation studies, cells were preserved in culture media containing 5% DMSO or CS5 (5% DMSO solution). for hypothermic storage studies, cells were combined with culture media or HTS-FRS and stored at 2-8°C. To simulate a physically induced-stress during transport, liquid and gel cell samples were placed into a styrofoam shipping/storage container and a force (stress) was applied during the storage. Recovery was assessed post-preservation. Results: for cryopreservation, the CS5 solution combined with gel resulted in a 13% and 16% improvement in viability for NHDF and HMSC cultures respectively. Interestingly, an ~20%
decrease in viability for both cell types was found when the gel was combined with culture media+5% DMSO. For hypothermic storage and stress studies, the gel solutions resulted in improved overall viability. Following a stress-induced application gel solutions resulted in a significant increase in NHDF viability (10% - media+gel; 20% - HTS-FRS+gel) compared to non-gel solutions. Overall, the HTS-FRS solution significantly improved recovery and viability regardless of cell type compared to cultures stored in growth media. Conclusion: In summary, the results of this study demonstrate that the incorporation of a gel during preservation can improve the stability and recovery of biologics. Methods to improve transport and preservation of may ultimately improve the overall integrity of the biologic.

1542/B700
Assessing the Toxicity of Nanoparticles using Caenorhabditis elegans as a Model System.
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C. elegans are excellent models for various biological studies. These small nematode worms have been used in several areas of research including developmental biology, neurobiology, oxidative stress, and aging. Some important characteristics of these worms as a model system include a small eukaryotic system, sequencing of the complete genome, short-life cycle and ease of maintenance and propagation in the laboratory. Nanoparticles are currently used in numerous applications including cosmetics, cleaning supplies, and medical preparations. The exact mechanism of toxicity associated with nanoparticle use is unknown. In study, we explored the use of C. elegans as a model system to assess the toxicity of nanoparticle exposure. The worms were exposed to nanosized fluorescent latex beads, blue-dyed latex beads, talc, or ultrafine silicon dioxide (SiO2). The update and translocation of the particles inside worms were confirmed using nanosized fluorescent beads. After one week of exposure, most of the beads were clustered in the body of the worms but also found dispersed throughout the worms. When C. elegans are limited by space and food, they are capable of entering into Dauer Stage until optimal living conditions are met. Following three weeks in the Dauer stage, beads were still found clustered in the body of the worms. Preliminary studies also suggest particle exposure affects the locomotion of the C. elegans. We observed the clumping of the worms and decreased movement following nanoparticle exposure. In conclusion, our studies to date support the use of C. elegans as a model system to assess the potential toxicity of nanoparticle exposure.

1543/B701
Dual Pulse Labeling using a New Thymidine Analog 5-ethyl-2'-deoxyuridine (EdU) to Detect Alterations in S-phase Progression by Fluorescence Microscopy and Flow Cytometry.
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The measurement of cellular proliferation is fundamental to the assessment of cell health, genotoxicity, and the evaluation of drug efficacy. Changes in DNA replication during S-phase can give insights into mechanisms of cell growth, cell cycle kinetics, and cytotoxicity. A common method for detection of cell proliferation is the incorporation of a thymidine analog during DNA synthesis. Incorporation of two different analogs at different time points can further define cell cycle kinetics. Traditionally the dual pulse method has been done by combining 5-bromo-2'-deoxyuridine (BrdU) with iododeoxyuridine (IdU) or chlorodeoxyuridine (CldU), using multiple BrdU antibodies that cross-react with either IdU or CldU for detection. The objective of this study was to examine an alternative dual pulse labeling method using the new thymidine analog, 5-ethyl-2'-deoxyuridine (EdU), with subsequent detection by click chemistry. Using quantitative flow cytometry, multiple BrdU antibodies were screened for their specificity for BrdU. While many BrdU antibodies cross react with EdU, no cross reactivity with incorporated EdU was observed when using the BrdU antibody clone, MoBU-1. Flow cytometry and fluorescence microscopy confirmed labeling of BrdU did not interfere with the detection of EdU, and that incorporated BrdU was not detected by click chemistry. Using this method for dual pulse labeling, we examined the effect of
several drugs with known biological effects such as DNA damage, cell cycle arrest, and apoptosis. Cells were pulsed with EdU, followed by drug treatment, and then pulsed with BrdU. Altered S-phase progression was observed after drug treatment, as evident from changes in the percentage of cells labeled with BrdU alone, EdU alone, or both EdU and BrdU. Evaluation of S-phase labeling patterns by fluorescence microscopy further defined cell cycle status of individual cells. In conclusion, this method eliminated concerns of antibody specificity associated when pulse labeling with two different halogenated deoxyuridine derivatives. Furthermore, dual pulse labeling with EdU and BrdU provided an accurate and simplified method of examining alterations in DNA replication during cell cycle progression.

1544/B702
Evaluation of Tau Phosphorylation using a Non-traditional Immunoassay Platform.
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Microtubule associated protein tau is actively phosphorylated at Alzheimer’s disease-related pathological epitopes. Many Tau epitopes are believed to be phosphorylated by various kinases. When a kinase inhibitor is introduced, phosphorylation can be monitored In Vitro utilizing an automated bioanalytical system (Gyrolab) that allows for development of immunoassays with sub-microliter sample volumes for accessing physiological and qualitative changes. Assay development employing a micro-fluidics platform determines specificity by the analyte-specific biotin-labeled capture antibody, which is used to functionalize the surface of streptavidin-coated particles (prepacked nanoliter columns within the structure). The detection antibody is fluorophore-labeled and the signal is acquired by laser-induced fluorescence. Results indicate analysis of specific phosphorylated Tau products using this platform further characterized the effects of modulating kinases. It is also noted that by treating embryonic rat cortical neuronal cultures with okadaic acid (a phosphatase inhibitor), an increase in phospho-tau threonine 181 was observed. Through enabling high throughput and reproducibility, opportunities to define biomarkers involved in the investigation of Alzheimer’s disease can be rapidly pursued, driving forward the development of new therapeutic agents.

1545/B703
A Comparison of a Homogenous Bead-based Amplified Luminescence Technology AlphaLISA and a Colorimetric ELISA for Detection of Human Matrix Metalloproteinase 9 in Complex Sample Matrices.
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Enzyme-linked Immunosorbent assay (ELISA) is a well known technique in the area of immunoassays. ELISA involves a number of wash steps, which makes it a laborious and time-consuming process. In the field of drug discovery, technologies which can give robust, sensitive, automatable and reproducible immunoassays at a lower cost are highly desirable. Amplified Luminescent Proximity-based Homogeneous Assay (AlphaLISA) is a bead-based homogenous immunoassay which can give comparable or even better results in less time, cost and sample volume. In this study, we compared ELISA and AlphaLISA platforms commonly used for immunoassays and examined performance (sensitivity, dynamic range, variability) as well as assay complexity, time to perform, and cost. The AlphaLISA matrix metalloproteinase 9 (MMP9) kit, EnVision multidetection instrument, and microplates were supplied by PerkinElmer. The colorimetric ELISA MMP9 kit was from a well-known supplier. The dynamic range, inter- and intra-assay variation was studied in spiked MEM + 10% FBS. Both the kits showed good reproducibility and accuracy. Cell culture samples containing MMP9 secreted upon PMA stimulation of U937 cells also showed similar results using the two kits. Using AlphaLISA, we can obtain a PMA dose-response curve of MMP9 induction without any sample dilution, even in RPMI + 10% FBS. AlphaLISA requires twenty times lesser sample volume and gives a five-fold wider dynamic range. A wider dynamic range allows the customer to measure most samples without any dilutions. Because the AlphaLISA kit does not require any wash steps, both the elapsed time and hands-on time required to perform the assay was much less than for the ELISA kit. Overall,
the AlphaLISA kit offered benefits over the ELISA kit of wider dynamic range, a significantly faster and easier protocol, a lower cost per well, and comparable performance in other respects.

1546/B704
Study of PKC-Dependent Cellular Signaling in a Spatial- and Temporal-Specific Manner by Photoactivatable Synthetic Compounds.

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Photo-activatable protecting groups have been demonstrated their compatibility in a cell biology field as tools for discovery of physiological functions such as cellular signaling. Protein kinase C (PKC) has 11 family members. Our research is focused on PKCδ, which is correlated to diverse diseases such as tumor progression and neurological disorders. This kinase is located on upstream of signaling for cell proliferation or transcription, thus it is difficult to detect the effects of ligand binding in time-resolved ways. It is known that the response to ligand binding could be detected by observing translocation in cytosol. Isozyme-specific ligands must be synthesized in a systematic manner. This structure-activity relationship approach is very powerful strategy for the discovery of optimized ligands, however, the mechanisms of cellular signaling occurred by ligand binding is still unclear. To tackle these problems, photo-activatable groups (6-bromo-7-methoxycoumarin (Bmc) and 6-brom-7-hydroxycoumarin (Bhc)) were attached to the ligands giving “caged compounds”. The synthetic caged molecules showed a 100- to 400-fold decrease in binding affinity for PKC, compared to the corresponding parent compounds. The effects on PKC translocation by the binding of compounds were assessed by confocal laser scanning microscopy. The results indicated that caged compounds have no effects on translocation of PKC and that the translocation occurs after photo-irradiation by specific wave lengths (365 nm for Bmc and 405 nm for Bhc). This method could be applied to detect the signaling which is caused by ligand binding in a spatial- and temporal-controlled manner. The present data could reveal details of cellular signaling correlated to PKC activation.

1547/B705
Precise Patterning of Human Mesenchymal Stem Cells to Investigate Spatial Dependence on Commitment.

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Commitment of mesenchymal stem cells to different lineages is regulated by various cues in the immediate microenvironment, such as individual cell shape and relative geometrical arrangement. Because the two are interdependent, each can be individually influenced by external factors. In Vitro such cell density and micro-patterning. The distance between cells will dictate what type of signaling is utilized (e.g., direct, paracrine, or endocrine), which will, in turn, determine the cells that take part in the resulting signal transduction. Therefore, it becomes crucial to establish a precise cell patterning technique to mimic the desired tissue or pathology to be studied, in order to gain truly relevant insight from artificial cell culture. Here we have developed a laser-based direct write system (MAPLE DW) to demonstrate precise patterning of mesenchymal stem cells into specific geometric configurations. This tool enables the effects of cell communication on the differentiation of human mesenchymal stem cells into osteoblasts or adipose tissue to be investigated. Using this technique we attempt to manipulate MSC commitment by precise patterns containing varying cell densities. Additionally, having the ability to pattern human mesenchymal stem cells into idealized cellular constructs, including co- and multi- cultures, can begin to answer questions about the specific parameters that directly impact the behavior of
hMSCs. The capability to accurately manipulate the positional direction and timing of hMSC differentiation can begin to shed light on the parameters needed In Vivo to treat injured and diseased tissues, illuminating the importance of these cells in tissue regeneration.

1548/B706
Mechanical Characterization of Primary Human Mesenchymal Stem Cells via Dual-Beam Optical Stretching.
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Optical stretching allows the mechanical properties of whole tissue cells to be measured in suspension via interaction with dual unfocused laser beams. We are applying this technique to study primary adult human mesenchymal stem cells, as mechanical behavior could provide a useful characteristic for studying and sorting these cells, enabling correlation with downstream mechanotransduction mechanisms. Here we present the average whole-cell creep compliance from a population of greater than six hundred cells as they are expanded In Vitro to produce a therapeutically relevant quantity. Techniques such as automated syringe rotation were developed to address the challenge of working with these relatively large, heavy cells. We have also documented the cell surface morphology (smooth, blebbing, and other presentations) because of the importance of successful edge detection by machine vision when analyzing optical stretching data. The average time-dependent strain profile is described well by an offset-power-law rheological model, adopted by others to describe the mechanical properties of tissue cells in the adherent, contractile state, where the power-law exponent for both the stretching and recovery periods is approximately 0.2. This experimental evidence for power-law behavior is the first to originate from a biomechanics measurement technique that does not involve physical contact with the cell.

1549/B707
Rapid Cell Labeling and Tracking with Designer Peptide Linkers.
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The utility of cellular labeling for molecular imaging of stem cells, angiogenesis, blood pool imaging, and immune activation is clear. We now report a generalizable labeling strategy that is applicable to a variety of cell types or synthetic vesicles that possesses a lipid mono- or bilayer coating such that a specially-designed short amphipathic peptide fused to a cargo (e.g., targeting ligand, imaging agent, drug) will insert stably into the membrane and function as a imaging reporter and/or therapy delivery vehicle. Methods and Results: to illustrate this novel labeling concept, a 19 amino acids peptide derived from a cationic melittin peptide (a component of bee venom) backbone was constructed and fused to a fluorophore (FITC) located at the amino terminus. Flow cytometry, fluorescence microscope, and surface plasmon resonance confirmed the stable insertion (and lack of toxicity) of the fusion complex into the membranes of synthetic liposomes, cultured macrophages, and isolated red blood cells (RBCs). Next, FITC-peptide loaded cells generated with simple one step incubation (1 hour), were injected In Vivo into athymic NCR-nu/nu homozygous mice. Whole body fluorescence images acquired with a Xenogen scanner demonstrated an immediate, disseminated, strong intravascular signal right after the injection. Conclusion: A variety of cell types can be rapidly and safely labeled with a selected cargo using this peptide linker approach. This strategy could be useful not only for cellular molecular imaging and tracking in regenerative therapies, but also for creating cell-based Trojan horse constructs for innate homing and site targeted delivery.
1550/B708

New Phosphorescent Oxygen-Sensitive Probes Based on Cell Penetrating Peptides.
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Similar to Ca^{2+}, ATP, ROS, NO and other metabolites which are analyzed routinely, molecular oxygen (O_2) is an important parameter for studies of cell bioenergetics, mitochondrial function and cellular responses to hypoxia. However, self-loading probes for non-invasive measurement of cellular O_2 are not widely available. Here, we report on a new class of O_2 probes comprising covalent conjugates of Pt-coproporphyrin (PtCP) with cell-penetrating peptides. The best results obtained with TAT-related oligoarginine peptide conjugates, which provide fast (6-16 hrs) and efficient loading of various types of mammalian cell lines (PC12, Hela etc) and primary cells, and can be directed to various subcellular compartments. The cells loaded with these new O_2 probes produce metabolic responses to stimuli of cell respiration that can be assessed by a time-resolved fluorescence plate reader. This allows simple and high-throughput measurement of local O_2 concentrations within the cell.

Molecular Biology (1551 – 1560)

1551/B709

Development of In-House Low Cost Laboratory Protocols for the Detection of Genetically Modified Food.
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In recent years, foods produced by genetic engineering technology have been on the world food market. However, with reported advantages, many risks and concerns of the use of such products have been identified such as their effects on human health, environment, etc. Therefore, biosafety aspects, regulations and labeling of these foods are still contentious issues in most countries. Thus, detection of Genetically Modified Organisms/Food(GMOs/F) plays a crucial role in developing regulations on GM foods. This study consists of several objectives including the selection of a suitable DNA extraction protocol, development of a direct GM plant identification PCR program, development of a quantitative estimation method using certified reference soya samples, screening of GM soya samples found in the Sri Lankan market and the screenings for structural genes (Bt-toxin and Roundup-Ready genes) in GM positive samples. According to the results, modified CTAB protocol was selected as the most suitable protocol for DNA extractions. A successful PCR program was developed for direct identification of GM plants using a universal plant primer and PCR sensitivity of this assay showed that up to 0.05mg/ml GM DNA could be detected. Unknown GMF sample was quantified using newly developed quantification assay and it contained approximately 1.7% GM. Based on the market survey, out of 20 tested samples, only four samples were GM positive showing that GM products are present in the local market and that one of these products was labeled as “non-GM”, while other three did not have any label indicating their GM status. According to results of screening for structural genes, Roundup-Ready genes were positive in the GM positive samples while all were negative for Bt-toxin gene. The methods thus developed are cheaper than existing methods and market surveys pose important safety issues, which require the enforcement of legislative guidelines on labeling and appropriate traceability systems in order to guarantee public health and consumer choice.

1552/B710

Rapid Screening of Antibody Aggregates and Protein Impurities Using Short Gel Filtration Columns.
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Gel filtration (GF) is an excellent tool to acquire information about sizing (identity), purity and specially the multimeric state of the protein of interest. To speed up the analysis and keep sample and buffer consumption at a minimum, prepacked short GF columns have been developed, Superdex™ 200 5/150 GL and Superdex 75 5/150 GL. With lengths of only 15 cm and volumes of 3 ml, these columns allow rapid analysis (6-12 min/run) with minimal sample (4-50 µl) and buffer consumption. This work shows three different applications using these short gel filtration columns. Three samples of one purified antibody from earlier optimization work using HIC, were analyzed on Superdex 200 5/150 GL. In only 45 min the sample containing the lowest amount of antibody dimers was identified. Another affinity purified protein contained co-purified truncated protein. Using Superdex 75 5/150 GL, the estimation of percentage of truncated protein was done in 12 min. Fast purity check of two Strep-tag II proteins was done using GF and in 12 min results similar to SDS-PAGE analysis was obtained.

1553/B711
Stabilization of RNA for Laboratory Analysis, Shipment and Biobanking.
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Objective: to develop a physical matrix to stabilize RNA, during use as a laboratory reagent, and to enable the (safe) shipping & storage of RNA specimens at ambient temperature. The value of RNA as a tissue analyte has been the subject of persistent technical concern, due to its short half-life In Vivo and in vitro. In the lab, instability results from RNA’s profound susceptibility to hydrolysis, both enzyme and metal catalyzed. Under more extreme conditions, such as long-term storage or at high temperature, RNA also becomes susceptible to non-hydrolytic chemistry, especially oxidation. Here, we describe an approach to the physical stabilization of RNA, based on an inert, water-soluble mineral matrix, which includes a cocktail of RNase inhibitors and antioxidants, referred to as GenTegra-RNA. During routine use, a freshly-purified RNA solution is added directly to GenTegra-RNA, which had previously been coated as a film matrix on the bottom of a plastic tube or well. Following brief mixing to dissolve the matrix, we show here that the resulting GenTegra-RNA solution imparts about an order-of-magnitude increase in RNA stability during routine fluid-phase, room temperature storage, allowing RNA to become stable over at least 8 hours of laboratory manipulation at ambient temperature, removing the need to work on ice. GenTegra-RNA matrix has been designed to stabilize RNA under non-routine, non-standard laboratory conditions, such as during exposure to elevated temperature or during long-term ambient temperature storage: similar to those incurred during un-refrigerated “book rate” sample shipping, or biobanking in the absence of cryogenics. That second set of protective attributes is obtained by allowing a GenTegra-RNA solution to passively air dry, significantly reducing the rate of RNA hydrolysis, due to complete removal of water, and reducing RNA oxidation, via protective RNA entrapment inside the resulting solid mineral matrix. Data are shown to demonstrate both short-term, fluid phase, ambient temperature RNA stabilization and long-term RNA stabilization, in the air-dried phase, under extreme conditions, for RNA specimens purified from a number of frozen and fixed tissues.

1554/B712
A Novel Feeder-Free Embryonic Stem Cell Culture System That Supports Mouse Embryonic Stem Cell Growth, Proliferation, and Pluripotency.
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Standard embryonic stem cell culture systems require co-culture with mitotically inactive mouse embryonic fibroblast feeder cells (iMEFs) to maintain their undifferentiated proliferative state. While iMEFs provide a suitable attachment surface and crucial soluble factors promoting embryonic stem cell (ESC) growth and proliferation, iMEFs are time-consuming to prepare. More importantly, iMEFs vary from lot-to-lot and contaminate ESCs with carryover iMEFs from previous culture passages. These latter shortcomings confound basic research attempting to dissect the culture components or underlying gene and protein expression patterns important for ESC
proliferation and differentiation. Development of a feeder-free system for embryonic stem cell culture using a synthetic matrix would provide a ready-to-use substrate which is consistent from lot-to-lot without iMEF carryover. iMEFs make abundant amounts of hyaluronic acid (HA) which is important for not only embryogenesis but also for human embryonic stem cell culture (1). Using mouse embryonic stem cells (mESCs) as a model system, we reasoned that the use of an HA-rich matrix would provide a suitable starting point for preparing a novel feeder-free substrate for undifferentiated growth. Here we report the use of a chemically defined, crosslinkable HA-based substrate (HyStem-CTM) for feeder-free propagation of mESCs in the presence of FBS. mESCs plated on HyStem-C maintain excellent morphology and offer comparable plating efficiency to those grown on iMEFs. Data from flowcytometry analysis as well as immunocytochemistry confirms the presence of key recognized pluripotency markers Oct3/4 and SSEA1. This novel feeder-free cell culture system has potential uses in proteomic analysis since no carryover iMEF proteins will be present in embryonic stem cell extracts. In addition, statistics from high content analysis and high-throughput screening efforts employing mouse embryonic stem cells will be improved due to increased consistency during the screening campaign. Finally, the possibility exists to alter the stiffness or composition of the matrix in a manner that may enhance efforts to drive the pluripotent cells down desired lineages (2).

1555/B713
A New System for Recombinant Protein Expression in Pichia pastoris. J. E. Fletcher, W. Kudlicki; Life Technologies, Carlsbad, CA

Pichia pastoris is commonly known for its capacity to secrete high levels of recombinant protein, yet its use as an expression system has been limited by the lack of convenient methods for cloning and screening of recombinant strains. To circumvent these limitations, a new P. pastoris expression system is introduced that supports rapid cloning into several novel Pichia expression vectors. All the vectors contain the alcohol oxidase I (AOX 1) promoter for methanol-induced expression and the complete adenine 2 (ADE2) gene to provide a robust auxotrophic selection of integrated clones when transformed into P. pastoris ade2Δ strains. The new vectors and ade2Δ strains are used to demonstrate secretion and/or intracellular expression of three model proteins; Calmodulin (CalmL3), green fluorescent protein (GFP) and human serum albumin (HSA). The use of protease deficient ade2Δ strains are shown to reduce degradation of secreted CalmL3. Finally, the application of this system for the production of functional mammalian GPCRs is illustrated. The availability of these new technologies vastly improves cloning efficiency and enables the easy transfer of thousands of available genes for recombinant protein expression in P. pastoris.

1556/B714
Multiplexed Bead-Based Proteomic and Genomic Analyses of Inflammatory Biomarkers in Human Skin Model: A Method for the Evaluation of Dermatological Drugs. R. Streeper1, E. Izbicka1, A. Diaz1, D. Campos1, A. Powell2, A. Zhang3, B. Yee3, T. Yao3, R. Chuang3, L. Pastor3, T. Nguyen3, M. Nguyen3, N. Ogawa3, L. Si AS3, 1BTNS LLC, Marion, TX, 2The University of Texas Health Science Center at San Antonio, San Antonio, TX, 3Affymetrix Inc., Fremont, CA

Objective: to evaluate anti-inflammatory activity of a range of topical drugs and determine biomarker signatures of their actions in a human skin model using multiplexed proteomic and genomic tools. Methods: We evaluated regulation of inflammatory biomarkers in a human 3-dimensional skin model using irritants [croton oil, capsaicin, 1-chloro-2,4-dinitrobenzene (CDNB), lipopolysaccharide (LPS), sodium dodecyl sulfate (SDS)] and counterirritants (indomethacin, dexamethasone, diphenhydramine, doxycycline, all-trans retinoic acid, 4-ethyxybenzaldehyde, salicylic acid, azelaic acid, green tea extract, and a novel alkyl azelate preparation HF1107). After topical exposure to irritants and counterirritants used either alone or concurrently for 18 hours, we measured levels of ATP, 45 cytokines and chemokines and prostaglandin PGE-2 in cultured media and tissue lysates. Tissues were examined microscopically for responses to treatment.
Inter-group differences were assessed by Student’s t-test. Results: Multiplexed immunoassays and QuantiGene Plex assays indentified “signature patterns” of both protein and RNA markers based on highly significant (t < 0.001) differences. We scored anti-inflammatory activity of test articles using significant >2-fold inhibition of pro-inflammatory analytes including IL-1b, IL-6, IL-8, IL-12p40, IL-17F and IL-23. The patterns of up- and downregulated analytes were distinct for all examined groups. SDS and CDNB were the strongest irritants. Relative potency of counterirritants followed the order: HF1107 > green tea extract > indomethacin > dexamethasone.

Conclusions: This study ranked relative activities of test articles on modulating local dermal inflammatory response in skin and provided a proof of concept for using multiplexed screening of diverse dermatologic agents. Parallel evaluation of test article activity on select target expression at the gene and protein level has offered deeper mechanistic insights than either method alone.

1557/B715
Improved Methods for Lentivirus Purification and Concentration.
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Current commercially available methods for lentivirus purification can be problematic in that they generally rely on treated membranes that are used as an anion exchange substrate. Loading the crude viral supernatant onto these matrices require syringe or vacuum assemblies to push or draw the sample through the membrane. This force can potentially shear the virion resulting in decreased yields and purity (average elution yield of 52%, n=5 by qRT-PCR). Our method utilizes an anion-exchange resin packed into an SPE column and relies on gravity for flowrate. The gravity purification format demonstrated timely completion with the overall process requiring 45 minutes and resulting in an average yield of 78% (n=6). This method also showed increased purity by SDS-PAGE Silver Stain when compared with treated membrane purifications using the same virus preparations. In certain applications, such as in-vivo or high MOI transductions, high concentration, low-volume preparations are beneficial. Attempts to scale up low-yield lentiviral production methods can result in lentivirus that is too dilute for the intended application with a volume too large to be easily concentrated by ultracentrifugation. Our scalable lentiviral concentration method utilizes a 1 to 4 hour incubation followed by a short spin in a low speed centrifuge. This half-day process results in up to 100-fold concentration of VSV-G pseudotyped lentivirus and also shows increased purity over crude supernatant by Silver Stain. We have used this scalable method to concentrate up to 250ml of low-titer lentivirus supernatant ~100-fold concentration as measured by FACS. This reagent has also been used to achieve exceptionally high final viral concentrations; LVX-ZsGreen1 has been concentrated to titers up to 1E+10 IFU/ml (FACS). We have also improved the scalability of our column purification by concentrating the lentivirus sample prior to purification. 100ml of LVX ZsGreen1 supernatant was concentrated to 10ml and then column purified and eluted in 3ml; resulting in a 56% yield by FACS titration. These two methods used together or separately provide increased utility for Lentivirus post-production processing.

1558/B716
An Innovative Tool to Reveal Mechanisms of Transcriptional Regulation.
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Understanding the mechanisms of transcriptional regulation is vital to interpret gene expression changes in a biological context. Although many different bioinformatics approaches have been proposed to discover mechanisms of transcriptional regulation, they require significant effort and time to identify each of the candidate regulators under physiological conditions. To overcome this problem, a novel reverse-genetics technology has been developed to probe the involvement of multiple transcription factors for regulating expression of specific genes in a parallel fashion. This technology combines multiplexed siRNA and real time quantitative PCR methods. These arrays feature siRNAs specific for transcription factors pre-dispersed and stabilized into the wells of 96-well cell culture plates. In this study a cancer focused panel, consisting of siRNAs for 84 transcription factors known to play important roles in cancer biology, was used to identify which
transcription factors are involved in regulating the expression of CDKN1A, FAS and TP53INP1 in MCF-7 cells after treatment with 5-fluorouracil (5-FU). The results confirmed that TP53 and SMAD4, both known down-stream modulators of 5-FU, play central roles in regulating expression of CDKN1A, FAS and TP53INP1 under the influence of 5-FU. In addition, the array also identified the involvement of the vitamin D receptor (VDR) in regulating gene expression under the influence of 5-FU. These findings present opportunities to understand the functional and drug resistance mechanisms of 5-FU in cancer therapies. Moreover, these results validate the efficacy and performance of this methodology to rapidly and accurately determine the transcription factors involved in regulating multiple genes under physiological conditions.

1559/B717
A Toolbox for Ab Initio 3-D Reconstructions in Single-Particle Electron Microscopy.
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Structure determination of a novel macromolecular complex via single particle electron microscopy depends upon overcoming the fundamental challenge of establishing a reliable 3-D reconstruction using only 2-D images. We have developed a “toolbox” of ab initio reconstruction techniques that provide several options for calculating 3-D volumes in an easily managed and tightly controlled work-flow. This toolbox is designed to streamline the reconstruction process by removing the necessity for bookkeeping, while facilitating transparent data transfer between different software packages. It currently includes procedures for calculating ab initio reconstructions via random or orthogonal tilt geometry, tomograms, and common lines, all of which have been tested using the 50S ribosome. In addition, the techniques have been applied to the low-resolution structural characterization of Spindle Assembly Protein 6 (SAS6), hypothesized to compose the inner nonameric array of the centriolar hub. The ring-like structure predicted by the image-processing algorithms within our toolbox reveals a novel architecture adopted by proteins having coiled-coil motifs and provides the first 3-D representation of the basic building blocks of the centriole.

1560/B718
Proteome-level Changes in Folding and Assembly within Stressed Cells Probed by Cysteine Shotgun Labeling.
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Protein unfolding, disassembly, and aggregation underlie many diseases, but detailed study of these processes in intact cells has been limited. Cysteine labeling with multiple cell-permeable fluorescent dyes leads to staged labeling of exposed cysteine residues, and subsequent tandem mass spectroscopy (MS) of the adducts ultimately reveals changes in protein structure and association due to stress in whole cells or cell lysates. To identify the most susceptible proteins and Cys sites in a rigorous manner, we have begun to thoroughly map changes in whole-cell lysates under native versus urea-denaturing conditions and as a function of time and temperature (37-60°C). In SDS-PAGE gels, 16 prominent bands were selected with MS identifying >1,000 distinct proteins with labeled Cys. An initial analysis of a 50 protein subset that includes talin, filamins, nonmuscle myosins, spectrins, and more, focuses on exposure kinetics as deduced from ion flux peak integration and spectral counts. Structural data from cysteine dual-labeling agrees with other sources such as solvent-accessible-surface-area calculations from crystal structures and individual protein studies. Specific sites in Filamin a and B, Talin 1, Myosin 9, alpha-actinin-4, heat shock protein 1 and elongation factor 2 appear especially amenable to further study in intact and stressed cells.
**Epithelia I (1561 – 1572)**

**1561/B719**

The Growth Promoting Effect of KGF on Limbal Epithelial Cell is Mediated via Upregulation of ΔNp63α through p38 Pathway.

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Corneal epithelial stem cells are thought to reside in the limbus, the transition zone between cornea and conjunctiva. The stemness of these cells is thought to be regulated by a variety of intrinsic and extrinsic factors, including the niche extracellular matrices and the paracrine/autocrine factors present in the niche milieu. Keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) have been suggested to be candidate paracrine factors that regulate the proliferation, migration, and differentiation of the limbal epithelial cells, however, the underlying mechanisms are still poorly understood. In an ex vivo limbal explant culture, we found that KGF is a more potent growth stimulator for the epithelial outgrowth than HGF. Immunofluorescence studies of the epithelial outgrowth harvested from HGF or KGF treated culture showed that the expression patterns of keratins 3 and 14 were essentially similar. In contrast, p63 was highly expressed in KGF treated limbal epithelial sheets but not that of HGF. Kinase inhibitor studies showed that the KGF-induction ΔNp63α is mediated via p38/Sp1 pathway. The response of limbal epithelial cells to KGF-induced growth stimulation was significantly reduced when ΔNp63α expression was silenced by siRNA transfection. Our findings clearly indicated that stimulation of the epithelial outgrowth from limbal explants by KGF is dependent on the expression of ΔNp63α. Since KGF is a known paracrine factor secreted by limbal keratocytes, our findings strongly suggest that limbal keratocytes may regulate limbal epithelial cell growth and differentiation through a KGF paracrine loop with ΔNp63α expression as one of it’s down stream target.

**1562/B720**

Epithelial Prosecretory Mitogen Lacritin is Protective Against Non-Apoptotic and Non-Necroptotic Cell Death with Autophagy by Targeting FOXO3a Apparently Independent of PI3K.

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Lacritin is a 12.3 kDa prosecretory mitogen secreted mainly by lacrimal acinar cells, with contributions from ocular surface cells and by the salivary gland. Lacritin’s C-terminus targets an N-terminal heparanase-dependent domain in syndecan-1 (Ma et al, J. Cell Biol. 174:1097-1106, 2006) to trigger rapid NFAT/mTOR mitogenic signaling (Wang et al, J. Cell Biol. 174:689-700, 2006). Deletion of 25 C-terminal amino acids (C-25) from lacritin is inactivating. Since inflammatory cytokines INFγ and TNF are augmented in dry eye, we asked whether lacritin is protective against cell death. Recombinant human lacritin and negative control C-25 were generated in E. coli and purified. Human corneal epithelial (HCE; Riken) cells were treated with TNF alone, or with INFγ followed by TNF in the absence or presence of lacritin or C-25, and HCE cell viability was assayed. Lacritin survival signaling was studied by phosphoblotting and with pharmacological inhibitors AKT-IV, STO-609, wederolactone, EGCG, U0126, rapamycin and cyclosporine. Autophagic flux was examined using vinblastine and leupeptin. All reagents were optimized via dose response and time course analyses. Sensitization with INFγ followed by treatment with TNF promoted a form of cell death coincident with LC3 lipidation. Death was respectively unaffected by apoptosis and necroptosis inhibitors zVAD and Nec-1, and was without DNA laddering although cleaved PARP was evident. Death was almost completely blocked by lacritin, but not negative control C-25. This effect was coincident with diminished LC3 lipidation and sudden FOXO3a phosphorylation in a biphasic dose-dependent manner with a 10 nM lacritin.
optimum. Lacritin cell survival activity is inhibited by FOXO3a kinase inhibitors AKT-IV and wedelolactone, but not by EGCG, STO609 or negative control U0126. Lacritin is protective against epithelial cell death in a biphasic dose dependent manner with a lacritin optimum of 10 nM. It appears to do so by rapidly promoting the phosphorylation of FOXO3a thereby retaining this pro-death transcription factor in the cytoplasm, and by blocking autophagy. Whether death is by autophagy, or only coincident with autophagy, remains to be determined. Supported by EY018222 (GWL).

1563/B721
Distribution of 14-3-3 Proteins in the Human Eye Cells and Tissue.
S. Dimitrijevich, J. Shankardas, R. Wordinger; UNTHSC, Fort Worth, TX

Background: The expression of 14-3-3 sigma isoform (stratifin), was reported to be epithelial cells specific in several human tissues. The studies of possible involvement of stratifin in cancers of epithelial cell origin have found variability of expression that questions the specificity of expression. The eye is an organ with several different epithelia that present a unique opportunity to address questions of the epithelial cell specificity of stratifin. Objective: to examine the expression of 14-3-3 proteins in ocular epithelial tissue and primary cells and cell lines with specific focus on the expression of stratifin. Methods: The immunohistochemical and immunocytochemical analysis was carried out on hydrated sections obtained from paraffin embedded human eye tissues. The expression in the epithelia of the lens (LE) ciliary process (CP) and retinal pigmented layer (RPE) was determined. The western blot analysis was performed on the primary lens epithelial cells (LEC), retinal pigmented epithelial cells (RPE) and cell line, non-pigmented ciliary process epithelial cell line (NPCE) and primary trabecular meshwork (TM) cells and cell line. Results: All the 14-3-3 isoforms were expressed in the TM tissue, primary and cell line except stratifin. Similar response was observed for RPE and its primary cells and cell line. Non-pigmented ciliary process epithelium and lens epithelium also did not express stratifin but all the other isoforms of the 14-3-3 family were expressed. The expression of the 14-3-3 family of proteins is very similar in the corresponding tissues. Conclusions: In the human eye the expression of stratifin appears to be confined to the stratified epithelia.

1564/B722
Extracellular Role of Secreted 14-3-3 Sigma (Stratifin) in the Human Cornea.
J. Shankardas, S. Dimitrijevich; UNTHSC, Fort Worth, TX

Background: Stratifin secreted by primary keratinocytes was found to up-regulate MMP-1 in dermal fibroblasts. We have reported that corneal and conjunctival epithelia cells secrete 14-3-3 gamma and zeta in addition to stratifin. We are interested in the mechanisms involved in these paracrine effects of stratifin and other secreted isoforms. Objective: to show membrane association of stratifin and 14-3-3 zeta and gamma in corneal epithelial cells and determine how the intercellular signals are initiated in corneal fibroblasts (keratocytes). Methods: The localization of stratifin, 14-3-3 gamma and zeta was determined by indirect immunofluorescence using isoform specific antibodies followed by total internal reflection (TIRF) microscopy. Cellular uptake of stratifin in target fibroblasts was determined by western blot analysis. Results: 14-3-3 gamma, zeta and stratifin are expressed intracellularly but are also associated with the cell membrane as shown by TIRF microscopy. Confocal microscopic analysis shows that stratifin co-localizes with EGFR in corneal epithelial cells. In keratocytes treated with conditioned medium from corneal epithelial cells there is membrane association of stratifin as determined by TIRF analysis. Cell lysates from these cells were negative for the presence of stratifin as determined by Western blot analysis. Conclusions: We showed by TIRF microscopy that 14-3-3 gamma, zeta and stratifin localize to the corneal epithelial cell membrane. We are in the process of determining if the association is with the inner or outer leaf of the membrane. The absence of entry of stratifin into the keratocytes suggests that the activation of MMP-1 takes place via as yet unknown receptor
mechanism. Colocalization of stratifin with EGFR in corneal epithelial cells suggests that EGFR could be a possible target receptor in these cells.

1565/B723
Odontoblast TRP Channels and Thermo/Mechanical Transmission.
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Odontoblasts function as a mechanosensory receptor because of the expression of mechanosensitive channels in these cells. However, it is unclear if odontoblasts direct the signal transmission evoked by heat/cold or osmotic changes. This study investigated the effect of heat/cold or osmotic changes on calcium signaling and the functional expression of the thermo/mechanosensitive transient receptor potential (TRP) channels in primary cultured mouse odontoblastic cells using RT-PCR, fluorometric calcium imaging, and electrophysiology. TRPV1, TRPV2, TRPV3, TRPV4, and TRPM3 mRNA were expressed but TRPM8 and TRPA1 mRNA was not. The receptor-specific stimulation of TRPV1-3 (heat-sensing receptors) and TRPV4/TRPM3 (mechanic receptors) caused increases in the intracellular calcium concentration. Moreover, the channel activities of TRPV1-4 and TRPM3 were confirmed using a whole-cell patch-clamp technique. These results suggest that primary cultured mouse odontoblasts express heat/mechanosensitive TRP channels and play a role in the underlying mechanisms of thermo/mechanosensitive sensory transmission. This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A084007).

1566/B724
A Mechanism for Organizing and Maintaining Lineage-Specific Domains In Mammary Gland.
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In adult tissues, multi-potent progenitor cells are some of the most primitive members of the developmental hierarchies that maintain homeostasis. That progenitors and their more mature progeny share identical genomes, suggests that fate decisions are directed by interactions with extrinsic soluble factors, ECM, and other cells, as well as physical properties of the ECM. Utilizing a highly-parallel combinatorial microenvironment functional screening platform in combination with 3D organotypic culture systems we previously demonstrated that multipotent human mammary progenitor cells are surprisingly flexible in their cell fate decisions in response to different microenvironments. A number of the putative cell-extrinsic determinants of cell fate that were identified in our screens were juxtaposed to the stem cell zones in vivo, but were not necessarily inside of them, suggesting that there should be a means by which progenitors can experience different microenvironments in vivo. To determine how lineages sort relative to one another, we have cultured together mixtures of differently labeled lineages from primary human mammary epithelial cells (e.g. myoepithelial, luminal, or progenitor) inside of micropatterned geometrically regular micro-wells. Mixtures of cells were observed over time, with and without perturbing influences, and changes in spatial distributions of cells from different lineages were quantified. Here we have identified a general mechanism by which mammary epithelial cells from the myoepithelial, luminal epithelial, and progenitor lineages are sorted In Vivo to maintain tissue structure and homeostasis.
Background: Colony stimulating factors (CSFs) act on stem cells leading to lineage specific differentiation. SCF and GM-CSF stimulated cells that undergo an "epithelial-to-mesenchymal" (EMT) transition acquiring many important stem cell characteristics. Although evidence suggests that these factors may be candidate treatments for liver injury, the role of CSFs in intrahepatic biliary epithelium remodelling is unknown. Thus, our aims were to characterize the molecular mechanisms of specific CSFs that regulate the stemness phenotypes of cholangiocytes from human and mouse bile ducts. Methods: The expression of CSFs receptors (EPO, G-CSF, GM-CSF) was studied in normal human cholangiocyte (HiBEC and H69) lines, and small and large murine cholangiocyte cultures (SMCCs and LMCCs) by immunocytochemistry and real-time PCR assay. Cell proliferation was quantitated using MTS assay. The progression of EMT was monitored through the mesenchymal marker S100A4 by real-time PCR analysis and immunoblots. Results: All CSFs receptors were expressed to a similar degree in all cholangiocytes tested. The combination of SCF (10 ng/ml) + GM-CSF (100 ng/ml) induced a 2.3 ± 0.3-fold increase (p < 0.01) of the proliferation index when compared to SMCCs treated with SCF. Furthermore, quantitative PCR analysis revealed a 3.8 ± 0.5-fold increased expression of S100A4 after SCF plus GM-CSF administration in SMCCs compared to SCF alone (p < 0.01), suggesting that the inducible stem cell properties in this cell line. In contrast, no significant changes were observed in the proliferation index and S100A4 expression in the human and LMCCs by other combinations. The SCF plus GM-CSF treatment also significantly activated Erk and Akt dependent signaling pathways in cultured SMCCs, the pathways which play the central roles in the self-renewal division of stem cell populations. Summary and Conclusions: SMCCs have an intrinsic higher potential than the other bile duct epithelial cells to respond to SCF plus GM-CSF, confirming the potent role of this combination in the development and remodeling of the intrahepatic biliary epithelium. These data support further study of SCF plus GM-CSF as the adjuvant therapy in hepatobiliary injury.

1568/B726
Mechanics-driven Spontaneous Pattern Formation.
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The development and functionality of tissues depends on the morphology of individual cells. However, it is not yet understood what morphogenetic patterns cells spontaneously develop in relation to environmental mechanical properties and how these patterns influence tissue development and functionality. Meanwhile, the process of tissue morphogenesis is mostly known at the initial and final states; much less characterized are intermediate state dynamics. By changing substrate rigidity, adhesiveness, and surface topology through micro-fabrication techniques, we observed that cells develop different morphogenetic patterns in response to environmental mechanical changes. In particular, cells develop oscillatory bipolar, tripolar, quadropolar, to multipolar shapes as the surface rigidity and adhesiveness increases. The selection of patterns also has a strong dependence on the surface topology. Using pharmacological inhibitors, we found that Rac1, PI3-K, MLCK, and ROCK are involved in such morphogenetic pattern formation. A three-component phenomenological lateral inhibition model has been developed to account for the underlying mechanisms with predictions agreeing well with the experimental data. Analyzing the dynamics of actin structures using multi-photon microscopy further suggests that intermediate-state dynamics during the formation of these patterns play an important role in tissue morphogenesis. Moreover, these morphogenetic patterns are observed at both single- and multi-cell levels, suggesting that the single-cell pattern formation is collectively retained at the multi-cellular level and serves as the fundamental unit to regulate and integrate tissue morphogenesis as a whole.
Role of Nesprin-2 in Cell Migration and Tissue Repair.
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Nesprins are multifunctional proteins anchored in the nuclear envelope through their highly conserved C-terminal KASH-domain. Through alternative splicing and alternate promoter and/or terminator usage the Nesprin genes can give rise to a multitude of differently expressed isoforms varying in their domain architecture and length. The largest isoform of Nesprin-2, Nesprin-2 Giant, is composed of an N-terminal actin binding domain (ABD) followed by several spectrin repeats and the KASH domain. We are characterising a Nesprin-2 Giant knock out mouse. In this mouse part of the ABD of Nesprin-2 was deleted. The knock out mice showed interfollicular epidermal thickness which might be due to an increased epithelial nuclear size compared to wild type. Primary dermal fibroblasts and keratinocytes showed nuclear deformations. Furthermore, Nesprin-2 Giant knock out fibroblasts have a defect in cell migration and cell polarity compared to wild type cells. In the current study we investigated the role of Nesprin-2 in tissue repair by an In Vivo wound healing assay. We found that also In Vivo wound healing is altered in the knockout mice compared to wild type mice and that there is a significant delay in healing during new tissue formation and tissue remodeling phase.

Ligand Activation of EphA2 Regulates Mitogenic Signaling Pathways Involved in Human Keratinocyte Differentiation.
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EphA2 is a receptor tyrosine kinase (RTK) that becomes activated by ephrin ligands residing on adjacent cell surfaces. In general, Eph-RTKs and ephrins are found at tissue boundaries and function to segregate discrete cell populations during development. However, EphA2 and ephrin-A1 are co-expressed in a variety of epithelial cells where their function remains poorly characterized. Primary human epidermal keratinocytes provide a good model system to study epithelial EphA2 activation since these cells also express abundant ephrin-A1 but downregulate these juxtamembrane signaling complexes upon terminal differentiation. These observations prompted us to test whether EphA2 transmits signals between neighboring keratinocytes that control entry into a differentiation pathway. We first determined that EphA2 was recruited to nascent cell-cell contact sites along with the calcium-dependent adhesion molecule, E-cadherin, by switching keratinocytes from 0.03 mM to 1.2 mM calcium in order to stabilize intercellular junctions. Gene silencing studies showed that EphA2 activation was coincident with and furthermore required for MAPK/Erk1/2 suppression in keratinocytes undergoing a calcium switch. In order to more specifically activate EphA2 in the absence of other juxtamembrane signaling complexes, keratinocytes were maintained in low calcium and treated with a recombinant ephrin-A1 ectodomain peptide (efnA1-Fc) or an Fc control. Peptide ligand activation of EphA2 not only suppressed Erk1/2 signaling but further led to the rapid internalization and downregulation of this RTK. Moreover, the accelerated loss of EphA2 resulted in a marked increase in keratinocyte differentiation, as assessed by elevated levels of epidermal structural proteins such as desmoglein 1 and keratin 10. In contrast, sustaining EphA2 levels by ectopic expression impaired the ability of keratinocytes to undergo terminal differentiation in a 3-D raft model of the human epidermis. Collectively, our studies reveal a key role for EphA2 in mediating epithelial cell-cell communication during keratinocyte differentiation.

Calcium Ion Propagation in Cultured Keratinocytes and Other Skin Cells in Response to Hydraulic Pressure Stimulation.

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2009 ASCB Regular Abstracts
Epidermal keratinocytes express various receptors, which are activated by temperature, osmotic pressure, mechanical stress, and chemical stimuli, and we have proposed that these receptors mediate skin surface perception. In the present study, we examined intracellular calcium responses to external hydraulic pressure. First we compared the responses of undifferentiated and differentiated normal human epithelial keratinocytes. We then observed the responses of fibroblasts, umbilical vein endothelial cells, and lymphatic endothelial cells, which are also present in skin, to show the special profile of keratinocytes for the sensory of skin. Cells cultured on glass-bottomed dishes were loaded with fura-2 acetoxymethylester, an intracellular calcium indicator, then stimulated with hydraulic pressure controlled by a microinjector. Changes of intracellular calcium concentration were represented as values of fluorescence intensity ratio measured with a fluorescence microscopy system. Elevation of intracellular calcium was observed in response to pressure in keratinocytes, fibroblasts and vein endothelial cells. The calcium propagation extended over a larger area and continued for a longer period of time in differentiated keratinocytes as compared with the other cells. The response of the keratinocytes was drastically reduced when they were incubated without calcium in the medium. Application of a non-selective TRP (transient receptor potential) channel blocker also reduced the response. These results suggested that differentiated keratinocytes are sensitive to external pressure and that TRP might be involved in the mechanism of this response.

1572/B730
Phospholipase C-delta1 Regulates Hair Keratin Expression and Hair Shaft Formation Downstream of Foxn1.
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Phospholipase C (PLC) is a key enzyme of phosphoinositide metabolism. PLC hydrolyzes bioactive lipid, phosphatidylinositol 4,5-bisphosphate and produces other bioactive lipid, inositol 1,4,5-Trisphosphate and diacylglycerol. PLC-delta1 is one of PLC isoymes and highly expressed by epidermal keratinocytes. To elucidate the physiological functions of PLC-delta1, we generated PLC-delta1 knockout (KO) mice. PLC-delta1 KO mice showed marked hair loss associated with epidermal hyperplasia. The appearance of a nude mouse is very similar to that of a PLC-delta1 KO mouse and hair shafts of both mice are bent and fail to penetrate the epidermis. In nude mice, the gene encoding the transcription factor Foxn1 is spontaneously mutated, and this mutation leads to insufficient hair keratin expression and abnormal hair shaft structures. Since exogenous expression of Foxn1 induces upregulation of PLC-delta1 and PLC-delta1 expression is markedly decreased in skin of nude mice, Foxn1 seems to function as an upstream regulator for PLC-delta1 expression in hair follicles. We generated PLC-delta1 KO mice that have PLC-delta1 gene driven by Foxn1 promoter. In these mice, defects of hair keratin expression and hair shaft structure are completely rescued. We also generated nude mice that have PLC-delta1 gene driven by Foxn1 promoter and found that hair keratin expression can be partially rescued in these mice. These results strongly suggest that PLC-delta1 regulates hair keratin expression and hair shaft formation downstream of Foxn1. Now we are investigating the mechanisms by which PLC-delta1 regulates normal hair formation.
Minisymposium 17: Autophagy and Organelle Turnover (1573 – 1578)

1573
Mannose-6-phosphate Receptor Independent Pathways of Lysosome Biogenesis.
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Lysosomes are catabolic organelles that degrade extracellular material internalized via endocytosis and intracellular components sequestered by autophagy. Lysosomes contain two classes of lysosomal proteins: 1. the lysosomal hydrolases responsible for degradation and 2. the lysosome-associated membrane proteins (LMPs) that regulate lysosome integrity, acidity, fusogenicity and dynamics. Most newly synthesized lysosomal hydrolases are transported from the trans-Golgi network (TGN) to the endosomal system by mannose 6-phosphate receptors (MPRs), which recognize the hydrolases by their mannose 6-phosphate (M6P) moieties and package them into clathrin-coated transport vesicles. Lysosomal delivery of LMPs does not require the MPRs and various lines of evidence indicate that lysosomal hydrolases can also be transported via MPR-independent pathways. The molecular and morphological details of these MPR-independent pathways are largely unknown. Objectives: to characterize MPR-independent pathways for lysosomal delivery of lysosomal hydrolases and LMPs. Our approach comprises RNAi, live cell imaging, immuno-electron microscopy and correlative live cell-immuno-electron microscopy. Results: I-cell disease is characterized by reduced activity of the GlcNAc-1-phosphotransferase (Ptase) resulting in the absence of M6P modifications on newly synthesized lysosomal hydrolases. We investigated lysosome biogenesis in various cell types of I-cell diseased patients and tissues from Ptase knock out mice. Our findings indicate that loss of Ptase activity affects the lysosomal delivery of some but not all lysosomal enzymes in a cell-type dependent manner. Especially in secretory cells, loss of Ptase activity resulted in the accumulation of autophagolysosomes. In addition, our findings indicate that TGN-to-endosome transport of LMPs occurs via a novel type of carrier that forms independent of clathrin but requires the presence of the HOPS component Vps41 for lysosomal delivery. Conclusions: In addition to the MPR pathway, other cargo-specific pathways to the lysosome exist that operate in a cell-type dependent manner.

1574
A Gene Network Regulating Lysosomal Biogenesis and Function.
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The lysosome is a specialized cellular organelle dedicated to degradative processes. As degradative requirements of the cell may vary depending on tissue type, age and environmental conditions, we postulated the existence of a system allowing the coordination of lysosomal activity. Here we show that most genes of the lysosomal system are connected in a regulatory network, which we named CLEAR (Coordinated Lysosomal Expression And Regulation). Genes belonging to this network display a coordinated transcriptional behaviour and share a specific E-box type regulatory element at their promoter regions. CLEAR elements are also associated to promoters of genes encoding non-lysosomal proteins involved in lysosomal biogenesis and degradative pathways. We found that the CLEAR network can be predictive for the identification of novel lysosomal proteins. We also demonstrated that the HLH-leucine zipper transcription factor TFEB binds to CLEAR sites. Overexpression and microRNA-mediated silencing experiments showed that TFEB positively regulates the expression of target genes, thus acting as
a master regulator of the network. We also found that the intra-lysosomal storage of undegraded material determines TFEB activation by promoting its translocation from the cytoplasm, where it resides in resting cells, to the nucleus. TFEB overexpression results in a significant increase both in the number of lysosomes and in the ability of the cell to degrade complex molecules, such as glycosaminoglycans (GAGs) and the pathogenic protein causing Huntington disease. Overall, our findings unveil a gene network dedicated to cellular degradative pathways that could be an appealing therapeutic target to enhance cellular clearing processes in neurodegenerative diseases.

1575

*Drosophila* Dacinus Regulates Endocytic and Autophagic Trafficking.
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Autophagy modifies the metabolic status of cells and their responses to stress and developmental signals. Thus, it is not surprising that multiple inputs regulate distinct steps along the autophagic pathway. The requirement for functional endosomes during the maturation of autophagosomes generates a complex interplay between these two distinct pathways to lysosomes. Here, we describe dAcinus as a novel element in this regulatory network. In order to identify components that mediate endosomal trafficking, we undertook a genetic screen for abnormal eye pigmentation, which constitutes a sensitive readout of endocytic trafficking in *Drosophila*. In this screen, we identified mutations in the gene encoding the *Drosophila* homolog of Acinus. Its mammalian homologs have been implicated in RNA processing and chromatin fragmentation during apoptosis. Loss-of-function analysis of *dacn* revealed two distinct functions in *Drosophila*: First, *dacn* is required for stabilization of early endosomes, thus modulating levels of Notch and EGFR signaling during development. Second, loss of *dacn* interferes with cellular starvation responses by inhibiting autophagosome maturation. In *dacn* mutant cells, autophagosomes form but fail to acidify and the formation of autolysosomes is inhibited. By contrast, overexpression of dAcinus causes lethality due to enhanced autophagy. Genetic epistasis experiments indicate that *dacn* affects autophagy downstream or in parallel to the Tor pathway. Interestingly, dAcinus is primarily localized to the nucleus. Taken together, our data show that dAcinus is a novel regulator of endosomal and autophagosomal dynamics.

1576

Molecular Regulation of the Autophagy Function of Beclin 1.
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Beclin 1, the mammalian ortholog of yeast Atg6, plays an evolutionarily conserved role in macroautophagy. Gene knockout/knockdown studies in diverse model organisms have shown a role for Beclin 1 in survival during stress, development, lifespan extension, innate immunity, tumor suppression, and protection against neurodegenerative diseases. Beclin 1 is part of a Class III PI3 kinase (PI3K) complex that may function both in the initiation of early autophagosomal membrane formation as well as in autophagosomal maturation. Several Beclin 1-interacting proteins have been identified that are reported to function in the positive (e.g. hhAtg14, Ambra-1, Bif-1, UVRAG) or negative (e.g. Rubicon, Bcl-2 family members, UVRAG) regulation of autophagy. However, little is known about how post-translational modifications of Beclin 1 contribute to the regulation of its autophagy function and/or interactions with other members of the Beclin 1/Class III PI3K complex. New data from our laboratory will be presented describing post-translational modifications of Beclin 1 that alter its autophagy activity and association with other members of Beclin 1 complexes. Furthermore, these data will describe previously unknown links between mitogenic signaling pathways, the regulation of Beclin 1 function, and autophagy. Together, our data suggest that Beclin 1 is a central integration point between signal transduction pathways that regulate cell growth and the execution of autophagy.
1577
Control of Autophagy Initiation by Phosphoinositide 3-Phosphatase Jumpy.
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Autophagy is a phosphatidylinositol 3-phosphate (PI3P)-dependent intracellular degradative pathway critical for cytoplasmic homeostasis, cell survival, death and differentiation, and cell-autonomous defenses against pathogens. Anomalies in the autophagy pathway contribute to a wide range of clinical conditions including cancer, aging, degenerative diseases, infection and inflammation. Although the dependence on PI3P-generating enzymes has been well established in the context of autophagy, the effects of PI3P turnover remain to be determined. Here, we screened the enzymatically active members of the myotubularin family, PI3P phosphatases, for effects on autophagy, and identified Jumpy (MTMR14) as an important autophagy regulator. Jumpy knockdown by siRNA enhanced whereas Jumpy overexpression suppressed autophagy as seen by biochemical and microscopy assays. Jumpy was found on autophagic isolation membranes, where it inhibited PI3P-dependent events, recruitment of WIPI1 (Atg18) to autophagic structures and LC3 lipidation. Jumpy R336Q mutant, catalytically inactive and associated with centronuclear myopathy, was deficient in suppressing autophagy. This work reports the first PI3P phosphatase directly involved in control of autophagy and reveals the previously unknown link between autophagy and centronuclear myopathy. (This work was supported by grants from NIH to VD, INSERM, CNRS, Collège de France, and from Association Française contre les myopathies, Agence Nationale de la Recherche and Foundation pour la recherche Médicale to JL, and from the Deutsche Forschungsgemeinschaft to TP-C. This project was supported in part by the Dedicated Health Research Funds from the University of New Mexico School of Medicine to IV)

1578
ATG12 Conjugation to ATG3 Regulates Mitochondrial Homeostasis.
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ATG12, an ubiquitin-like modifier required for autophagy, has a single known conjugation target, another autophagy regulator called ATG5. We have uncovered that ATG12 is covalently attached to multiple protein targets and have identified ATG3 as a novel substrate for "12-ylation". ATG3 is the E2-like enzyme necessary for ATG8/LC3 conjugation to phosphotidylethanolamine (PE) during autophagy. ATG12:3 complex formation requires ATG7 as the E1-activating enzyme, and an autocatalytic function of ATG3 as the E2 enzyme, which results in the covalent attachment of ATG12 onto a single lysine on ATG3. Mutation of this lysine abolishes ATG12:3 complex formation. To uncover the biological functions of ATG12:3, we have reconstituted ATG3-deficient cells with wild type ATG3 and a non-conjugatable mutant (ATG3KR). Remarkably, disrupting ATG12:3 complex formation has no discernable effect on starvation-induced autophagy. Indeed, during nutrient starvation and rapamycin treatment, ATG3WT and ATG3KR-expressing cells exhibit equal rates of both LC3 lipidation (LC3-II) and turnover in the lysosome, as well as the robust proteolytic degradation of autophagy substrates like p62SQSTM. In contrast, upon disrupting ATG12 conjugation to ATG3, cells display significantly increased mitochondrial mass and reduced levels of mitochondrial degradation upon treatment with chemical uncoupling agents. Furthermore, ATG3KR cells display resistance to cell death mediated by the intrinsic apoptotic pathway and possess a highly fragmented mitochondrial network, a phenotype that closely resembles cells with established defects in mitochondrial fusion. Overall, these results support a previously unrecognized role for the ATG12:3 complex in regulating mitochondrial homeostasis and function; they also broach the novel idea that the ATG12 conjugation system

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directs a broader range of cellular functions beyond autophagosome formation. Based on our findings, we are currently dissecting if ATG12:3 is required to selectively target mitochondria for autophagic degradation (mitophagy), if it regulates mitochondrial fission/fusion, or both.

**Minisymposium 18: Cell and Tissue Mechanics (1579 – 1584)**

**1579**

**Membrane Tension and Crowding Pressure: The Multimodal Mechanism of the Mechano-sensitive Channel MscS.**

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MscS is a ubiquitous osmolyte release valve found in essentially all walled organisms and their organelles. E. coli MscS, the best understood representative, transiently opens in response to membrane tension during osmotic downshock. Then, if moderate tension persists, it enters a tension-insensitive inactivated state. Crystal structures of the homoheptameric MscS complex reveal the transmembrane domain connected through the narrow gate region formed by TM3 helices to the hollow cytoplasmic domain (cage). We explored the conformational space of MscS with the new computational ‘extrapolated motion’ technique in molecular dynamics and constructed models for the resting and open states. The closed-to-open transition involving straightening of TM3s and iris-like expansion of the transmembrane domain satisfies experimentally determined spatial parameters and conductance. Through analysis of multiple mutations we identified membrane-to-gate force transferring contacts between the peripheral TM1-TM2 helices and the core TM3s. Disruption of these apolar interactions strongly favors fast inactivation, thus compromising survival of bacteria under gradually imposed osmotic shock. Crowding agents (PEGs) added to the cytoplasmic side also lead to fast inactivation, and higher concentrations of crowders close the channel completely. Independently, simulations suggest that the cytoplasmic cage domain may flatten and close the main gate. Thus, initially considered solely as a size-limiting pre-filter, the cage domain also appears to act as a large-osmolyte pressure sensor. Not only can it discriminate solutes by size, it can disengage the channel when turgor pressure is created primarily by high-molecular weight cytoplasmic constituents. We conclude that lateral tension acting on the transmembrane domain and osmotic forces perceived by the cage all converge on the gate. Therefore, MscS is structurally designed to intrinsically reconcile the opening stimulus (membrane tension) with the inhibitory effect of macromolecular crowding pressure.

**1580**

**Flows and Tension during Cortical Polarization of the C. elegans Zygote.**

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Asymmetric cell divisions are essential for generating cellular diversity in developing organisms. During the first division of the C. elegans zygote, antero-posterior (AP) polarization is facilitated by an anterior-directed flow of the cortical actomyosin meshwork, which segregates fate determinants between the emerging domains. Although a local weakening of the posterior cortex appears to initiate cortical flows, their underlying physical principles and regulatory mechanisms remain poorly understood. To investigate the forces driving flow and their regulation within the cell, we sought to understand the relationship between cortical flows, active contractility and cortical tension. To this end, we developed a UV laser ablation assay to measure cortical tension in the living embryo in a location- and direction-dependent manner. We find that in the direction orthogonal to flow (across the AP axis) cortical tension differs between the anterior and posterior domain, and is under the control of the Rho-GTP cycle. We furthermore reveal an anterior anisotropy in tension, with high cortical tension across the AP axis, but two-fold lower tension.
along the AP axis (the axis of flow). In contrast, cortical tension is isotropic and high throughout the cortex when polarization and flows are impaired, demonstrating that tensile anisotropy is a direct consequence of the underlying flow. Surprisingly, cortical tension along the AP axis is the same in the anterior and the posterior domain, i.e. flows are not associated with tension gradients. A quantification of myosin density and flow profiles together with a theoretical analysis reveal that cortical flows in the C. elegans zygote operate in a regime where the cortex is sufficiently viscous to support long-range cortical rearrangements by local contractions, which is vital for robust polarization. Our results suggest that the requirement to generate flow along the entire cell length produces tensile anisotropies without necessarily involving tension gradients. We propose that these mechanisms are not only fundamental to the establishment of AP polarity in the C. elegans embryo, but also to other developmental processes which rely on contractile flows.

1581
Mechanically Gated Ion Channels in Drosophila Morphogenesis.
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Dorsal closure is a model system of epithelial cell sheet morphogenesis in Drosophila embryos that requires actomyosin-based contractile forces in two adjacent epithelial tissues, the amnioserosa and lateral epidermis. As the forces that drive closure are resilient and coordinated in space and time, we hypothesize that they are regulated at the cellular level by a mechanosensory circuit. We present the following pharmacological, biophysical and genetic evidence in support of a role for mechanically gated ion channels (MGCs) in force regulation during dorsal closure. We find that: (1) Microinjection of either the peptide toxin GsMTx-4 or gadolinium (GdCl₃), inhibitors of MGC function, disrupts wild type dorsal closure in a dose-dependent manner, indicated by abnormal cell shapes, tissue behaviors, and failure to close. (2) Blocking MGCs via GsMTx-4 also compromises the ability of remaining epithelial tissues to upregulate tension following laser microsurgery protocols in which the tension generated by the amnioserosa is removed. (3) Tissue specific RNAi-mediated knockdown of MGC mechanosensor candidates reveals three distinct loci representing two families of MGCs which partially phenocopy the effects of GsMTx-4 and GdCl₃. Defects due to RNAi knockdown and/or mutant alleles include: failure to close, failure to undergo wildtype cell shape changes, failure to initiate epidermal seaming and delayed closure. Defects are more severe when RNAi is expressed in the amnioserosa, rather than the lateral epidermis, suggesting some tissue specificity for MGC function. Furthermore, embryos expressing RNAi against these candidates fail to recover from laser microsurgery, indicating an inability to redistribute forces for the resumption of closure. We conclude that MGCs provide a critical regulatory function for dorsal closure. This research contributes to our understanding of the molecular mechanisms that integrate mechanical feedback with force production to drive successful development. Grant Support: HD 040372-09 Training Program in Developmental Biology to G.L.H. and GM33830 to D.P.K.

1582
Mechanotransduction in the Merkel Cell-Neurite Complex, a Conserved Vertebrate Touch Receptor.
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The sense of touch is critical for hand dexterity that allows mammals to recognize and grasp objects. Different qualities of touch are encoded by sensory neurons with distinct properties. For example, shapes and fine textures are encoded by Merkel cell-neurite complexes, which mediate slowly adapting type I (SAI) responses. Merkel cells, which cluster in fingertips and other highly touch-sensitive skin areas, are enigmatic epidermal cells first described in 1875. The role of these
cells in touch has been debated for 40 years. Based on morphology, Merkel cells are proposed to be mechanosensory cells. If this model is correct, Merkel cells should 1) transduce force into membrane-potential changes that gate voltage-activated ion channels and 2) signal afferent neurons through synaptic transmission. Functional studies testing these predictions in intact skin have produced conflicting results. To address these questions, my laboratory combines mouse genetics, In Vitro systems and intact electrophysiological recordings. Our In Vitro studies have demonstrated that Merkel cells are intrinsically force-sensitive and that voltage-activated channels open downstream of mechanical stimuli. Using genomics, we have identified hundreds of highly enriched Merkel-cell transcripts. Strikingly, many encode neuronal transcription factors, ion channels and synaptic proteins. Collectively, these results suggest that Merkel cells are capable of serving as touch receptor cells and pave the way to discover transduction mechanisms. To determine whether Merkel cells are necessary for touch responses in the intact skin, we generated conditionally targeted mice that selectively and completely lack Merkel cells in the body skin. We then used an ex vivo skin-nerve preparation to survey the classes of touch-sensitive afferents in the saphenous nerve. Although we found no significant differences in nociceptive sensory fibers, we observed a complete loss of SAI responses among light-touch receptors in mice lacking Merkel cells compared with wild-type mice (P<0.02, Fisher’s exact test). These results demonstrate that Merkel cells are required for appropriate sensory coding of light touch.

1583

Uncovering a Postnatal Remodeling of Cochlear Hair Cell Apical Circumferential Shape Driven by Hair Bundle Morphogenetic Cues.

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Mechanical forces and associated mechano-sensitive processes play central roles in both the differentiation and physiology of hair cells. A so far unnoticed remodelling of the apical junctional complex (AJC) of cochlear outer hair cells (OHCs) parallels in these cells the appearance of their electromotility properties, by which they amplify the sound-evoked vibrations of the hearing organ. Driven by the idea that this shape transition might complement the function of OHCs as electromechanical transducers, we undertook a systematic study of the morphological changes affecting their apical circumference during early postnatal development. Using hair cell contour detection based on immunostaining of the AJC protein ZO-1, parametrized and analyzed by Fourier interpolation, we characterized the transition as a switch from a rounded-hexagonal to a non-convex circumference comprising two lateral lobes flanking a region of negative curvature on the cell’s neural side. A polarised redistribution of molecules that participate in the shaping of apical cellular junctions accompanied this switch, in particular immunodetection of Myosin II isoforms revealed their depletion from the region of the lateral lobes under formation. Correlation analysis further demonstrated that the non-convex shape adopted by the OHC apical circumference tightly fits the overlying V-configuration of the stereociliary bundle. Contour shape detection applied to mutant OHCs developing fragmented hair bundles, or with abnormal stereocilia arrangement and lengths, revealed a failure of the shape transition, which correlated with the degree of hair bundle disorganization. In these mutants, the redistribution of cortical proteins observed in wild type OHCs also failed to occur. Our results suggest an active mechanism in which internal cortical tensions under control of the nearby hair bundle reshape the OHC apical circumference. This transition, by impinging the mechanical coupling between the lateral membrane and the apical surface of OHCs in tight alignment with their hair bundle, may provide an ideal configuration to focalise forces generated by electromotility towards deflection of the stereocilia.

1584

Structure, Dynamics, and Elasticity of Cadherin-23 Repeats Involved in Hereditary Deafness.
The hair-cell tip link, an essential component of the mechanotransduction apparatus in the inner ear, has been proposed to be formed by cadherin-23 (CDH23) and protocadherin-15 (PCDH15). Both molecules belong to the cadherin superfamily of calcium-dependent cell-cell adhesion proteins and are mutated in hereditary deafness. Here we present x-ray crystallographic structures of the first and second extracellular cadherin repeats of CDH23, likely involved in heterophilic interactions with PCDH15. The structures show a typical cadherin fold for both repeats, but reveal a novel calcium binding site and an elongated N-terminus that precludes classical cadherin-cadherin binding interactions. The linker region between the repeats closely resembles a classical cadherin calcium-binding motif and contains the sites of certain mutations causing non-syndromic deafness (DFNB12). The new structures and molecular dynamics simulations begin to suggest ways in which CDH23 and PCDH15 bind end-to-end to form the tip link. Molecular dynamics simulations also suggest that deafness mutations and removal of calcium ions allow the unfolding of hair-cell tip links.

Minisymposium 19: Cell Migration (1585 – 1590)

1585
Regulation of Invadopodia by Extracellular Matrix Characteristics.
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Degradation of extracellular matrix (ECM) is important for migration of cells through tissues. Specialized cell structures called invadopodia are critical for ECM degradation and are formed by invasive, but not non-invasive cancer cells. Using modified ECM substrates, our recent data indicate that ECM is not only a passive substrate for invadopodia but also regulates the formation and function of these structures. Thus we find that physical ECM characteristics such as rigidity and crosslinking are critical modulators of invadopodia activity, with mechanotransduction signaling promoting invasiveness of cells. These data suggest a potential mechanism, via invadopodia, for the reported correlation of tissue density with cancer aggressiveness.

1586
Nanoscale Protein Organization in Focal Adhesions.
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Focal Adhesions (FAs) are organelles that cells use to sense and signal mechanical cues that control migration, proliferation, and differentiation. FAs are dynamic assemblies of >150 cytoplasmic proteins that link the cell cytoskeleton to the extracellular matrix (ECM) via clustered integrin transmembrane ECM receptors. Despite the importance of FAs for migration and mechanotransduction, the protein architecture of FAs remains unknown. We used interferometric PhotoActivated Localization Microscopy (iPALM), which localizes fluorescent proteins with better than 20 nm resolution in 3-dimensions, to determine the molecular scale organization of important proteins within FAs in fixed cells. Imaging U2OS cells expressing fusion constructs of FA proteins with Eos, a green-to-red photoactivatable fluorescent protein (FP), we found that FAs consist of a core region subjacent to the plasma membrane marked by the exclusion of actin and α-actinin.
The FA core interfaces with the actin cytoskeleton at a plane ~30-40 nm above the plasma membrane. Within the FA core, FA proteins were organized into well-defined axial stratifications. The cytoplasmic tail of αV integrin, focal adhesion kinase (FAK), and paxillin all localized to a narrow plane at ~5 nm above the plasma membrane. Vinculin, zyxin and VASP were localized in hierarchical layers centered at ~20, ~35, and ~45 nm, in that order, above the plasma membrane, spanning the FA core/actin junction. Using talin constructs with FP fused to the N- or the C-terminus, we found that the N-terminal talin head domain was anchored close to the plasma membrane while the C-terminus of talin extended up to the interface with the actin zone. Our results reveal for the first time the nanoscale protein architecture of FAs, which is based on talin forming an array of tethers that likely directly link the integrin cytoplasmic tail and actin, and which appears to be embedded in hierarchical layers of other FA components that may complement talin in reinforcing or regulating the ECM-cytoskeleton mechanical linkages.

1587
Regulation of Epithelial Cell Motile Behavior through Crosstalk between Extracellular Matrix- and Cell-Cell Adhesions.
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The pioneering studies of Abercrombie in the 1950's originally recognized that intercellular adhesion hinders cell migration. It remains unknown, however, whether this "contact-inhibition" results from the down-regulation of cell migration, its redirection, or both. During normal growth and development, epithelia undergo a large array of morphogenetic processes, from cohesive sheet migration to individual cell migration after dissociation. This suggests that crosstalk between cadherin-mediated intercellular adhesion and integrin-mediated cell migration on the extracellular matrix (ECM) may be more complex than simple inhibition upon cell-cell contact. To study the behavior of epithelial cells under controlled adhesion cues, we analyzed cell and membrane dynamics on micro-patterned surfaces designed with alternating stripes of ECM and adjustable amounts of E-cadherin. Addition of E-cadherin spatially confined integrin-based adhesions, globally dampened lamellipodia activity and biased cell migration parallel to the stripes, but cell migration rate was unaffected. However, depletion of alpha-E-catenin, a cytosolic actin-binding protein also associated with E-cadherin, increased both lamellipodia activity and migration rate as well as weakened E-cadherin-mediated adhesion. Conversely, selective depletion of the cytosolic pool of alpha-E-catenin increased migration rate but not lamellipodia activity, and did not affect adhesion strength. These results provide direct evidence that signalling by E-cadherin-mediated intercellular contacts dominates integrin-ECM adhesions and redirects, but does not down-regulate cell migration. Moreover, E-cadherin mediated adhesion down-regulates lamellipodia activity through a membrane-associated pool of alpha-E-catenin, whereas the cytosolic pool of alpha-E-catenin down-regulates migration rate in an E-cadherin adhesion-independent manner. Synergistic weakening of intercellular adhesion and up-regulation of cell migration by alpha-E-catenin depletion might govern morphogenetic processes that involve single cell migration. Conversely, redirection, but not down-regulation of the cell migration machinery upon E-cadherin-mediated adhesion may be crucial for cohesive tissue migration.

1588
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Cell migration requires formation of the dynamic leading edge which exhibits cyclical protrusions and retractions; however, the identity of pacemakers and cell signaling mechanism that controls those cycles has remained unknown. Here, we used live cell imaging of biosensors to monitor the spontaneous morphodynamic and signaling activities of epithelial cells and used correlative
image analysis to examine the role of PKA in protrusion regulation with single second temporal and micron spatial resolution. We report that duration of protrusion-retraction cycle is dramatically increased upon inhibition of PKA and that the activity of PKA at the tip of the leading edge closely coincides with the most rapid protrusion and with the activity of RhoA. This close correlation of RhoA activity and protrusions is maintained by PKA phosphorylation of RhoA at serine 188. Phosphorylation by PKA increases RhoA affinity to RhoGDI which results in sequestration of RhoA from plasma membrane and blockade of downstream signaling. Increased RhoGDI expression reverses the effect of PKA inhibition on protrusive morphodynamics and also corrects for the mistiming of activation of RhoA(S188A) that can not be phosphorylated by PKA. In summary, PKA phosphorylation of RhoA and its resulting effects on RhoGDI-RhoA interaction control the cycling of RhoA activity at the leading edge, the pacemaker of the protrusion-retraction cycle. These data provide mechanistic insight into how PKA, RhoA, and RhoGDI cooperate to control events at the leading edge of migrating cells that are involved in development, immune response or tumor invasion.

1589
Neutrophil Migration in a Transgenic Zebrafish Model of WHIM Syndrome.
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WHIM syndrome is a rare genetic disease characterized by neutropenia caused by the retention of neutrophils in the bone marrow and recurrent infections. The disease results from aberrant chemokine signaling due to a truncation of the CXCR4 chemokine receptor. We have developed a model of WHIM syndrome by generating transgenic zebrafish lines that express a GFP tagged zebrafish CXCR4 or WHIM truncated CXCR4 specifically in neutrophils. Neutrophils in WHIM-GFP embryos develop until 3 days post fertilization when they fail to enter the bloodstream, recapitulating the neutropenia seen in WHIM patients. Furthermore, live imaging of leukocyte trafficking shows that CXCR4-GFP expressing neutrophils readily respond to tissue wounding but the ability of WHIM-GFP neutrophils to respond to wounds is severely impaired although they maintain the ability to migrate randomly in the head and caudal hematopoietic tissue. Transient knock down of CXCL12, the ligand for CXCR4, in the WHIM-GFP transgenic line using morpholino oligonucleotides rescues the ability of WHIM-GFP neutrophils to respond to tissue wounding. This suggests that the inability of WHIM-GFP neutrophils to respond to wounds is CXCL12 dependent and suggests that CXCR4/CXCL12 plays a similar role in regulating neutrophil homeostasis in zebrafish embryos and humans. The WHIM-GFP transgenic line provides the first zebrafish model of a primary innate immunodeficiency.

1590
Molecular and Cellular Mechanisms of Vertebrate Eye Morphogenesis.
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Although the vertebrate retina is a well-studied paradigm for organogenesis, the morphogenetic mechanisms that carve the architecture of the vertebrate optic cup remain largely unknown. In early eye morphogenesis, retinal progenitor cells (RPCs) move first towards the midline, before turning around to migrate out into the evaginating optic vesicles. Neighbouring forebrain cells, however, converge rapidly and then remain at the midline. These differential behaviours are regulated by the transcription factor Rx3. We have identified a downstream target of Rx3, the Ig-domain protein Nlcam, and characterised its role in regulating cell migration during the initial phase of optic vesicle morphogenesis. Through sophisticated live-imaging and comprehensive cell tracking experiments in zebrafish, we show that ectopic expression of Nlcam in RPCs, as is
observed in Rx3 mutants, causes enhanced convergence of these cells. We propose that Nlcam forms part of the guidance machinery directing rapid midline migration of forebrain precursors, where it is normally expressed, and that its ectopic expression upon loss of Rx3 imparts these migratory characteristics upon RPCs. In a second morphogenetic event the hemispheric shape of an eye is formed. We analyzed the role of ojoplano(opo), a novel gene involved in the morphogenesis of epithelial tissues. Opo mutants in medaka fish, fail to fold the optic cup. We have characterized optic cup morphogenesis In Vivo and determined at the cellular level how opo affects this process. Opo encodes a developmentally regulated transmembrane protein that localizes to compartments of the secretory pathway and to basal end-feet of the neuroepithelial precursors. Opo regulates polarized localization of focal adhesion components to the basal cell surface. The ocular phenotype observed in opo is resembled by tissue-specific interference with integrin-adhesive properties. We propose a model of retinal morphogenesis that requires opo-mediated formation of focal contacts to transmit the mechanical tensions that drive the macroscopic folding of the optic cup.

Minisymposium 20: Functional Organization of Plasma Membranes (1591 – 1596)

1591
Myotubularin Roles in Muscle-Specific Membrane Compartmentalization.
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Myofibers are large multinucleated cells that rely on membrane specializations - t-tubules, sarcoplasmic reticulum and myotendinous junctions - to contract and maintain integrity. Regulation of membrane compartment identities is in part conveyed by phosphoinositide phosphates (PIPs), which are spatially-temporally regulated by dedicated kinases and phosphatases. Mutations in human phosphoinositide 3-phosphate phosphatase, Myotubularin1 (MTM1), underlie X-linked myotubular myopathy (XLMTM), suggesting tight regulation of PIP homeostasis is essential in muscle. Upon muscle-specific RNAi knockdown of Drosophila myotubularin (mtm), we found defects that mimicked XLMTM pathogenesis: mislocalization of nuclei, disorganization of myofibrils, onset of defects following myofiber differentiation and animal lethality. The stage of animal lethality correlated with detachment of persistent larval muscles (PLMs), large individual abdominal myofibers required for adult eclosion. Consistent with detachment of mtm depleted PLMs, βPS1-Integrin was absent from myotendinous junctions of detached myofiber ends and sarcomemmal costameres. Strikingly, βPS1 accumulation was detected on aberrantly large, internal membrane compartments, suggesting defects in integrin recycling to the sarcolemma. Dlg, a t-tubule resident protein, was similarly detected on large, internal membrane compartments of mtm depleted PLMs. The t-tubule system, a membrane network continuous with the sarcolemma, mediates excitation-contraction coupling. A reduction of transversal tubules was also observed upon mtm RNAi, implicating a role for mtm in t-tubule formation. The size and distribution of compartments detected by a marker for PI(3)P, a substrate for Mtm, were altered upon mtm knockdown. Importantly, animal viability and PLM morphological defects were rescued upon co-depletion of mtm with the class II PI3-Kinase, PI3K68D. Altogether, these results point to the significance of an mtm-dependent PI(3)P regulation in sarcomemmal organization, a possible relationship between t-tubule biogenesis and integrin trafficking, and the potential for discovery of modifiers in a fly model relevant to the decipherment of XLMTM pathology mechanism.

1592
A Novel Two-Tiered Model for Spectrin Function in Drosophila.
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Spectrin and ankyrin family members are implicated in several human diseases affecting neurons, muscles and erythrocytes. In the well-studied erythrocyte, spectrin and ankyrin form a
dense submembrane network that contributes to cell shape and membrane stability. Spectrin and ankyrin also are thought to function at high density in other cell types to organize specialized subdomains of the plasma membrane. Here we used genetic tools to directly test the requirements for β spectrin function using Drosophila as a model system. Unexpectedly, the results indicate that the essential function of Drosophila spectrin resides exclusively in the nervous system and that the amount of spectrin required to carry out function is far less than what is normally expressed in vivo. The Gal4-UAS system was used to knock down β spectrin in specific tissues with RNAi. Knockdown did not produce a lethal phenotype in tissues tested (epithelia, nervous system, fat body, etc.), even though antibody staining demonstrated RNAi efficacy. Nevertheless, the embryonic lethality of a chromosomal β spectrin mutation was overcome by nervous system-specific expression of a UAS β spectrin transgene driven by elav-Gal4. Thus, rescue to adulthood strictly depends on β spectrin expression in the nervous system. Interestingly, rescued adults appeared more fit when rescued with a low-expression transgene vs. A high-expression transgene, although lifespan was sharply reduced in all cases. Quantitative western blots demonstrated dramatic reduction (>8-fold) in spectrin abundance in the adult head when elav-Gal4 was used to drive β spectrin RNAi in the nervous system. Thus surprisingly little spectrin is required to carry out its essential function in vivo. Taken together the results suggest a novel two-tiered model in which some functions of the spectrin cytoskeleton require high protein density, but other functions do not. While only the low-density function appears to be required for viability in Drosophila, further evidence suggests that there are high-density functions as well, but they are redundant. We conclude that spectrin makes contributions to the formation of specialized membrane domains through mechanisms beyond scaffold formation.

1593  
Direct Interaction between Dynactin-4 and Ankyrin-B is required for Membrane Localization of the Dystrophin-glycoprotein-complex in Muscle Fibers.  
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Selective distribution and localization of membrane proteins to specialized membrane domains is a fundamental physiological requirement in metazoan cells. The cellular mechanisms underlying trafficking and localization of proteins to their site of action are to date not fully understood. We have recently described a novel ankyrin-based mechanism for functional organization of dystrophin and dystroglycan in skeletal muscle fibers (Ayalon et al. Cell 135, 1189-1200). We showed that ankyrin-B and ankyrin-G collaborate in a pathway for the localization and retention of the dystrophin-glycoprotein-complex (DGC) to two specialized membrane domains, skeletal muscle costameres and the neuromuscular junction. We also showed that ankyrin-B is required for a subset of microtubule-networks that are associated with these two membrane domains. In addition, dynactin-4, a component of the dynactin complex, can directly bind to ankyrin-B. We proposed a model where the dynactin complex, which associates with molecular motors and is required for microtubule-based trafficking of proteins and organelles, is required for trafficking of the DGC in an ankyrin-B dependent pathway. To establish our model we focused on the characterization of the interaction between ankyrin-B and dynactin-4. We now show that the direct interaction between ankyrin-B and dynactin-4 is a critical step in the localization of the DGC to costameres. Using In Vitro binding, and yeast two hybrid assays, we have identified the binding regions which are required for this interaction. Utilizing an In Vivo muscle transfection approach in mice, we knocked down the expression of either ankyrin-B or dynactin-4, and rescued with mutants of either ankyrin-B or dynactin-4, respectively, which are impaired in their ability to bind to the other protein. Results from these In Vivo structure-function experiments reveal that the localization of the DGC to costameres depends on the interaction of ankyrin-B and dynactin-4. As ankyrins, the dynactin complex and microtubules express ubiquitously in most if not all mammalian cells, the ankyrin-B - dynactin-4 interaction may be a key step in a general cellular pathway for targeting membrane proteins to specialized membrane domains.
The Functional Organization of the Axonal Membrane in Health and Disease.
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In vertebrate nervous systems a highly integrated network of neurons and glial cells works together to process and encode information through synaptic activity. This activity results in the generation and propagation of action potentials along axons. Neurons are highly polarized cells with distinct functional domains designed to receive synaptic input, to integrate electrical depolarizations, and to initiate and propagate action potentials. For example, neurons are roughly divided into two polarized domains: a somatodendritic and axonal domain. Additional levels of polarity exist within membrane subdomains: the axon initial segment and nodes of Ranvier comprise functional membrane domains where voltage-gated Na+ and K+ channels are highly enriched. These domains are central to action potential generation and propagation. In this talk I will present and discuss recent work demonstrating that both intrinsic and extrinsic (glial-derived) mechanisms contribute to the functional organization of the axonal membrane. For example, the intrinsic mechanisms depend on cytoskeletal and scaffolding proteins to 1) regulate the distinction between axonal and somatodendritic domains and 2) assemble and maintain ion channel clusters at initial segments. In contrast, assembly of ion channel clusters at nodes of Ranvier requires interactions between axons and their ensheathing glial cells. Finally, I will show that the cytoskeletal and scaffolding proteins found at axon initial segments are disrupted after nervous system injury leading to loss of ion channel clusters and neuronal polarity. These results suggest that loss of polarity and disruption of these functional membrane domains is a previously unrecognized consequence of injury.

Neurofascin Accumulation at the Axonal Initial Segment is Promoted by Neurotrophin Signaling and Doublecortin (DCX).
B. Winckler, C. Yap, M. Vakulenko; Neuroscience, University of Virginia, Charlottesville, VA

Many of the molecular players responsible for signal reception, integration, and propagation in neurons are spatially segregated. The cellular mechanisms ensuring the correct localization of molecules are fundamental to neuronal function and mislocalization of proteins can lead to disease. Neurofascin (NF) is a member of the L1 family of adhesion receptors and localizes to axonal initial segments (AIS) and Nodes of Ranvier, sites where action potentials are generated. NF is an ankyrin-binding protein, and ankyrin-binding activity by the conserved FIGQY motif is crucial to its localization on the AIS. When the tyrosine residue of the FIGQY motif is phosphorylated, ankyrin binding is greatly diminished and NF becomes highly diffusible. In support of this notion, we showed previously that the AIS-resident pool of NF is both non-diffusing and non-phosphorylated. In this work, we examine the potential role of doublecortin in targeting of NF. The lissencephaly-linked doublecortin protein (DCX) was previously found to bind the FIGQY motif of NF when phosphorylated. In neuroblastoma cells, the FIGQY motif is phosphorylated downstream of NGF signaling. We show here that NF targeting is increased by neurotrophin signaling in primary neurons. In order to determine if DCX function is on the AIS targeting pathway, we changed DCX levels in neurons: downregulation of DCX diminishes AIS targeting and DCX overexpression enhances targeting. The mechanism of the DCX effect on AIS targeting of NF is at least in part via increasing endocytosis: NF endocytoses poorly in PC12 cells, but its endocytosis is augmented by NGF and DCX expression. A DCX mutant incapable of binding clathrin adaptors does not augment NF endocytosis in PC12 cells and impairs NF targeting in neurons. In addition, inhibition of endocytosis with Eps15 mutants leads to decreased NF targeting to the AIS. These observations suggest that activity-dependent neurotrophic signaling at the nerve terminal might promote AIS targeting of NF.

Functional Organization of Ion Channels at the Axonal Initial Segment.
B. Dargent; INSERM UMR 641, Université de la Méditerranée, Marseille Cedex 20, France
The axonal initial segment (AIS) is a unique functional compartment of the neuron that is physiologically essential for the generation action potentials. The AIS also maintains neuronal polarity by forming a general membrane diffusion barrier that restricts the mobility of proteins and phospholipids. In addition, it contains a selectivity filter that controls the passage of axonal proteins. All these properties depend on the cytoskeletal adaptor ankyrin G that orchestrates AIS organization by concentrating Nav1 sodium channels, Kv7.2/Kv7.3 potassium channels and cell adhesion molecules like Neurofascin-186 and NrCAM. Our work aims for new insights into the functional organization of ion channels at the AIS and its molecular mechanisms. We showed that Nav1 targeting and clustering at the AIS is specified by an ankyrin-binding motif. Phosphorylation of this motif by Casein Kinase 2 (CK2) regulates Nav1 clustering and ankyrin G positioning, and therefore the assembly of the AIS. More recently, we examined whether ankyrin G controls the surface diffusion of interacting ion channels, using a single particle tracking method with quantum dots. In the absence of the general diffusion barrier (i.e. in young neurons), we observed that ankyrin G highly immobilizes ion channels at the cell surface via their direct interaction, and that channel immobilization is regulated by CK2 phosphorylation. We noticed that this immobilization by ankyrin G precedes the apparition of the general diffusion barrier, supporting a model in which the first step in the establishment of the diffusion barrier at the AIS is the immobilization of ankyrin binding proteins. This also confirms that CK2 acts in concert with ankyrin G to orchestrate the formation and the maintenance of the AIS.
**Minisymposium 21: Mitosis and Meiosis (1597 – 1602)**

1597  
**Molecular Recognition at the Kinetochore-Microtubule Interface.**  
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During mitosis, chromosomes connect to the plus-ends of spindle microtubules (MTs) through their kinetochores. Since incorrect kinetochore-MT attachments can lead to chromosome mis-segregation and aneuploidy, precise regulation of these interactions is critical. The NDC80 complex has been implicated as a linker between kinetochores and MT plus-ends, since it is required for kinetochore-MT attachment in cells and can directly bind MTs in vitro. How the NDC80 complex links kinetochores to MTs remains a debated issue. Here we describe a mechanism for binding that relies on both the intrinsically disordered Hec1 80 amino acid tail and the well-ordered Hec1 calponin homology (CH) domain. Using an siRNA-based silence and rescue strategy in PtK1 cells, we demonstrate that kinetochore-MT attachment mediated through the Hec1 tail domain is not based on molecular recognition of primary sequence-driven structural motifs, but is based on amino acid composition and charge. Specifically, cells depleted of endogenous Hec1 and rescued with Scr-Hec1, a Hec1 mutant in which the amino acids of the tail were randomized, generated stable kinetochore-MT attachments similar to cells rescued with wild-type Hec1. Additionally, we find that Hec1 tail-mediated attachment relies on a short domain for MT binding, as cells rescued with a Hec1 mutant containing a 25 amino acid tail were able to generate stable kinetochore-MT attachments similar to the wild-type condition. Binding mediated through this truncated tail is dependent on charge composition, as reduction of the positive charge by mutagenesis resulted in destabilization of kinetochore-MT attachments. Our findings also demonstrate that, in addition to the Hec1 tail domain, the Hec1 CH domain is required for mediating binding between kinetochores and MTs, as cells rescued with a Hec1 mutant containing a single point mutation on the outer surface of the CH domain fail to form stable kinetochore-MT attachments. Our findings suggest a molecular mechanism for NDC80-MT binding that relies on both non-primary sequence-based protein-protein interactions and interactions that conform to the sequence-structure-function paradigm, in which a well-defined 3-D structure is required for binding.

1598  
**The Importance of Augmin, and Mitotic Spindle-templated Microtubule Nucleation, in a Developing Organism.**  
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The formation of a robust, microtubule (MT)-based spindle apparatus capable of chromosome segregation occurs via multiple mechanisms, including centrosome- and chromatin-driven microtubule amplification. The conserved Augmin complex facilitates a third mechanism contributing to mitotic spindle assembly in animal cells, recruiting γ-tubulin to existing spindle MTs where it drives mitotic spindle-templated MT nucleation. Recent work from our lab identified 270 MT associated proteins (MAPs) from Drosophila embryos, 21 of which were shown to have essential roles in mitotic spindle organisation. Of these 21, seven encode known subunits of the Augmin complex. Reduction of any of the genes encoding these proteins in tissue culture cells leads to bipolar mitotic spindles possessing a reduced density of MTs in their central region. Here, we identify the MAP, Msd1 (Mitotic spindle density 1), as an eighth component of the Drosophila Augmin complex. We show that Msd1 localises γ-tubulin and the γ-TuRC subunit DGrip71 (NEDD1/GCP-WD) to mitotic spindles in both tissue culture cells and in the developing organism, and demonstrate directly that it promotes nucleation of MTs from within the mitotic spindle. Flies possessing a mutation in msd1 are viable, but female sterile. The primary defect in msd1 mutant embryos is an increase in mitotic spindle length during metaphase and a reduction
in mitotic spindle density, when compared to wild-type spindles. Crucially, however, although the Augmin complex is dispensable for viability, Augmin-dependent MT nucleation from within the spindle becomes essential in the absence of centrosomes; flies possessing mutations in both msd1 and centrosomin (cnn) are homozygous lethal, dying at an early pupal stage. Third instar larval neuroblasts from these cnn; msd1 mutants show ‘pro-metaphase like’ cells with weak, unorganised MT arrays emanating from mitotic chromatin, presumably reflecting the contribution of chromatin-directed MT amplification. Thus, (i) Drosophila possesses at least three mechanisms of MT organisation during mitosis and (ii) the Augmin complex has a crucial role in the development of the fly.

1599
MgcRacGAP is Required for Centromere Maintenance.
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CENP-A is required for centromere integrity and therefore crucial for kinetochore assembly and chromosomes segregation during mitosis. HsKNL-2\(^{M18BP1}\) is a DNA binding protein required for CENP-A localization to centromeres via an unknown mechanism. To better understand HsKNL-2\(^{M18BP1}\) function we co-immunoprecipitated HsKNL-2\(^{M18BP1}\) associated proteins from Hela cell lysates with two different specific antibodies directed against HsKNL-2\(^{M18BP1}\). Proteomic analysis and comparison to two non-specific control immunoprecipitations identified a subset of proteins specifically associated with HsKNL-2\(^{M18BP1}\). Among the specific proteins were HsKNL-2\(^{M18BP1}\), Mis-18, RbAp46, and RbAp48; a complex previously identified by similar methods. In addition, we identified the conserved GTPase activating protein MgcRacGAP as specifically interacting with HsKNL-2\(^{M18BP1}\). Immuno- and GFP-fusion localization studies showed that MgcRacGAP localized to centromeres in late G1, 6-8 hours after cell division. Interestingly, this localization pattern coincides with the completion of loading of new CENP-A. shRNA based depletion of MgcRacGAP resulted in \(~50\%\) reduction of CENP-A at centromeres indicating a mechanistic role in centromere specification. MgcRacGAP is required for cytokinesis, however we did not find a correlation between failed cytokinesis and CENP-A reduction, indicating that these two processes are likely independent. MgcRacGAP is antagonized by the GTP exchange factor ECT2 and together these proteins are thought to activate the small G-proteins Rac, RhoA and CDC42 via GTP cycling. We find that depletion of ECT2 or CDC42, but not Rac or RhoA, resulted in reduced CENP-A levels at centromeres. We therefore propose a model where GTP cycling of CDC42 recruited to centromeres in late G1 by HsKNL-2\(^{M18BP1}\) is required to maintain proper levels of CENP-A at centromeres. Alternatively, depletion of MgcRacGAP could lead to over activation of a particular G-protein and destabilization of CENP-A. We are testing these models by expression of constitutively active or dominant negative mutants and epistasis. In sum, these results highlight a new mechanism for centromere specification and maintenance.

1600
Kinetochore Dynein and Spindle Checkpoint Silencing in Human Cells.
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The dynein/dynactin motor complex localizes transiently to kinetochores and is proposed to silence the spindle checkpoint by transporting Mad1/Mad2 to the spindle poles following kinetochore-microtubule attachment. We are investigating the mechanism of checkpoint silencing by examining the conserved coiled-coil Spindly protein, which is essential for recruitment of dynein and dynactin to kinetochores. Spindly depletion abrogates poleward transport of Mad1/Mad2 and other corona-localized proteins, but does not prevent their removal from attached kinetochores. Accordingly, Spindly depleted cells proceed to anaphase with no apparent defects in chromosome segregation, albeit after a significant delay in chromosome alignment. Using an RNAi complementation system based on single-copy integrated transgenes, we identified a single amino acid mutation in a conserved motif in Spindly that does not perturb its
kinetochore localization but fails to recruit dynein/dynactin. The Spindly motif mutant largely rescues the chromosome alignment defect of Spindly depletions but, unlike wild-type Spindly, the mutant protein fails to be transported to spindle poles and is retained at attached bi-oriented kinetochores under tension. Persistence of the mutant Spindly protein at attached kinetochores is correlated with retention of Mad1/Mad2 at kinetochores, resulting in an extended metaphase arrest that is reminiscent of direct dynein/dynactin inhibitions. These results indicate that the primary reaction in checkpoint silencing in human cells is removal of Spindly by dynein/dynactin-mediated poleward transport. In the absence of Spindly, an alternative dynein transport-independent mechanism is capable of silencing the checkpoint and we speculate this mechanism is linked to the core microtubule-binding site of the kinetochore, whose components are critical for checkpoint activation. This alternative mechanism of silencing may dominate in organisms such as yeasts and higher plants that either lack kinetochore-localized dynein/dynactin or have lost dynein/dynactin entirely during their phylogeny.

1601 Phosphorylation-dependent Regulation of CENP-E by Aurora Kinases is Essential for Chromosome Congression.

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Centromere-associated protein E (CENP-E) is a processive, plus end-directed motor that powers chromosome congression and contributes to the capture and stabilization of spindle microtubules by kinetochore. CENP-E is highly phosphorylated during mitosis, which raises the possibility that phosphorylation may regulate the function of CENP-E at individual kinetochores. Strikingly, blocking phosphorylation of a single conserved site close to the motor domain of CENP-E prevents chromosome alignment with few chromosomes remaining close to spindle poles. Using purified components and a phospho-specific antibody, we demonstrate that Aurora kinases, both a and B, phosphorylate this site both In Vitro and in vivo. Phosphorylation occurs while CENP-E is bound to the kinetochore and reduces the affinity of CENP-E for microtubules in vitro. Moreover, CENP-E phosphorylation is shown to be required, downstream of Aurora activation, for the congression of incorrectly attached chromosomes that are moved to the spindle pole. Our findings demonstrate that CENP-E is under direct control of Aurora kinases to promote chromosome biorientation and to correct microtubule attachment errors that would otherwise lead to chromosome missegregation and aneuploidy.


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In fungi, the monopolin complex prevents certain types of improper kinetochore-microtubule (MT) attachments, both in mitosis and meiosis. In Saccharomyces cerevisiae, monopolin binds kinetochores in meiosis I and enforces co-orientation of sister chromatids; while in Schizosaccharomyces pombe, the complex inhibits merotelic attachment of individual kinetochores in mitosis and meiosis II. In both species, monopolin also has a nucleolar function in interphase, where it contributes to ribosomal DNA (rDNA) silencing and repeat stability. How monopolin accomplishes such seemingly disparate tasks in these very different molecular contexts is unknown. Using a combination of x-ray crystallography, electron microscopy, and supporting biophysical techniques, we have examined the architecture of the conserved core of monopolin from both S. cerevisiae (the Csm1 and Lrs4 proteins) and S. pombe (Pcs1 and Mde4). The S. cerevisiae Csm1/Lrs4 complex adopts a distinctive V-shaped structure, with two globular ‘heads’ spaced ~10 nm apart. These domains share a fold with Spc24/Spc25 of the conserved outer-kinetochore Ndc80 complex, suggesting a common evolutionary origin and related
functional properties. Further, we demonstrate a direct physical interaction between the globular head domains of Csm1/Lrs4 and the MIND/Mis12 complex, a conserved inner kinetochore subcomplex. Together, this evidence supports a model in which Csm1/Lrs4 acts as a bivalent clamp, cross-linking multiple MIND/Mis12 complexes and thereby co-orienting MT-binding elements both within and between kinetochores. We envision a similar structural mechanism for monopolin in the nucleolus, where Csm1/Lrs4 could bind multiple rDNA repeats to assist silencing and suppress unequal recombination.

Minisymposium 22: Stress Responses (1603 – 1608)

1603
Allesteric Activation of IRE1 via a Novel Ligand Binding Pocket.
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The unfolded protein response (UPR) couples the stress caused by a mismatch between the load of unfolded/misfolded proteins in the endoplasmic reticulum lumen and the chaperone reserve in that compartment to the activation of genes that restore protein folding homeostasis in the ER. The kinase-endoribonuclease IRE1 initiates signaling in the most conserved branch of the UPR by promoting the unconventional cytoplasmic splicing of a target mRNA whose spliced form encodes a potent transcription factor (Hac1p in yeasts and XBP1 in animals) that activates many UPR target genes. IRE1’s most conserved effector function thus consists of sequence specific endoribonucleolytic cleavage of an RNA target. This effector function is subordinate to the level of ER stress by a cascade that initiates with the stress-induced dimerization/oligomerization of IRE1’s lumenal domain, progresses through the trans-autophosphorylation of the kinase domain, which promotes high affinity nucleotide binding, and, in turn, stabilizes the activate dimeric/oligomeric state of the endoribonuclease. Here we report on the discovery of a novel ligand binding pocket in yeast IRE1’s cytoplasmic effector domain that can be engaged by a hydrophobic small molecule. Ligand binding to this pocket enhances IRE1’s endoribonucleolytic activity In Vitro and In Vivo and potentiates the effect of nucleotide binding at IRE1’s conventional nucleotide binding cleft. A co-crystal structure of IRE1’s effector cytoplasmic domain engaged by both by ADP at the nucleotide binding site and ligand at the novel pocket provides a plausible mechanism for ligand-induced IRE1 activity. The structure also defines various contacts between IRE1 and ligand at the novel pocket and reveals structural constraints on ligand binding. Rational mutagenesis of IRE1 supports the functional relevance of the structural model to IRE1 activation by this ligand and suggests the possibility of developing tools to explore the possible engagement of this pocket by endogenous ligands and other small molecules that may tune the IRE1 branch of the UPR independently of the state of the ER lumen.

1604
Membrane Expansion Alleviates Endoplasmic Reticulum Stress Independently of the Unfolded Protein Response.
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Cells adjust the sizes and shapes of their organelles in response to changing conditions. Here, we examine endoplasmic reticulum (ER) membrane expansion during the unfolded protein response (UPR) in the yeast Saccharomyces cerevisiae. We find that membrane expansion occurs through the generation of ER sheets, requires UPR signaling and is driven by lipid biosynthesis. Uncoupling ER size control and the UPR reveals that membrane expansion alleviates ER stress independently of an increase in ER chaperone levels. Converting the sheets of the expanded ER into tubules by reticulon overexpression does not affect the ability of cells to
cope with ER stress, showing that ER size rather than shape is the key factor. Thus, increasing ER size through membrane synthesis is an integral yet distinct part of the cellular program to overcome ER stress.

1605
A Ribosome-anchored Chaperone Network That Facilitates Eukaryotic Ribosome Biogenesis.
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Molecular chaperones assist cellular protein folding as well as oligomeric complex assembly. In eukaryotic cells, a network of chaperones, termed CLIPS, is transcriptionally and physically linked to ribosomes. Several CLIPS chaperones have been implicated in de novo folding. Here we show that a CLIPS network comprising the ribosome-anchored J-proteins, Jjj1 and Zuo1, also mediates the biogenesis of ribosomes themselves and the subsequent ribosomal export from the nucleus. Jjj1 and Zuo1 have overlapping but not identical functions in this complex process that involves the coordinated assembly and remodeling of dozens of proteins on the ribosomal rRNA. Jjj1 regulates the Hsp70 SSA and appears to play an important role in the nucleus where it associates with immature 60S ribosomal precursors and assists the processing of rRNA. Zuo1, together with its Hsp70 partners Ssz1 and SSB, also associates with ribosomal precursors and participates in rRNA maturation. Our results demonstrate that, in addition to their known role in de novo protein folding, some ribosome-anchored CLIPS chaperones play a critical role in ribosome biogenesis.

1606
A New Role for the Ubr1 E3 Ubiquitin Ligase in Cytoplasmic Quality Control.
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Protein quality control mechanisms exist in multiple compartments within the cell to selectively degrade misfolded proteins. Previous studies have shown that molecular chaperones play a role in the degradation of cytoplasmic quality control substrates, and that these misfolded proteins are disposed of via the ubiquitination-proteosome pathway. However, the ubiquitin ligases (E3s) that mediate chaperone-dependent ubiquitination are unclear. We sought to discover these ligases in S. cerevisiae, using genetic, molecular and biochemical means. Sequential screening revealed that two E3s, Ubr1 and San1, are important for cytoplasmic quality control. Both are dependent on the action of molecular chaperones. San1 has been shown to be involved in nuclear quality control, and its involvement may be dependent on a chaperone-mediated transport pathway. Ubr1 has been well studied in the N-end rule pathway. Our studies now reveal that this highly conserved ligase is responsible for ubiquitinating a broad range of misfolded substrates in the cytoplasm. Ubiquitination by Ubr1 requires cytosolic molecular chaperones and is specific for misfolded variants of the substrates studied. Furthermore, we have found that Ubr1 is physiologically important in this function, promoting survival of several proteotoxic stresses. Importantly, the quality control function of Ubr1 was not dependent on its previously studied role in the N-end rule pathway, but rather is a novel action of this E3. Ubr1 is a highly conserved ligase, found in all eukaryotes. Thus our findings allow for the interpretation of Ubr1 function in the broader context of its role in eukaryotic proteostasis, in normal cell life and the many pathophysiological conditions that involve misfolded proteins.

1607
Genome-wide Translational Profiling of the eIF2α-mediated Stress Response with Single-Nucleotide Precision.
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Cells respond to diverse stresses by altering gene expression at the level of protein translation, but it has been difficult to make quantitative and comprehensive measurements of translational control. We have developed a technique, called ribosome profiling, that enables genome-wide investigation of translation with single-nucleotide resolution. We performed ribosome profiling on yeast subjected to amino acid starvation stress, a treatment that induces the phosphorylation of the general translation initiation factor eIF2α. The highly-conserved eIF2α phosphorylation response causes a general decrease in protein synthesis coupled with an increase in the translation of specific stress response target genes, and our experiments reveal the global profile of this regulation. In both yeast and mammals, translational induction of phospho-eIF2α target transcripts is mediated by the presence of short translated sequences upstream of the protein coding-gene, called upstream open reading frames (uORFs). We were able to measure ribosome positions with single-nucleotide resolution and thereby directly measure translation of hundreds of uORFs. We also observed other sites of upstream translation indicative of wide-spread initiation at non-AUG codons. This unexpected non-AUG initiation was induced after amino acid starvation. As eIF2α has a direct role in start codon recognition, we hypothesize that its phosphorylation may drive the changes in initiation at non-AUG codons. This alteration in the stringency of initiation codon selection would result in differential regulation of transcripts based on the sequence of their 5’ untranslated region and could thereby play a role in the eIF2α-mediated translational response. We have adapted ribosome profiling to mammalian cells, in which there are four stress-responsive eIF2α kinases, and present preliminary measurements of In Vivo translation in higher eukaryotes.

1608
The Proteostasis Challenge: Stress, Aging, and Diseases of Protein Conformation.
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The health of the proteome depends upon the ability of the proteostasis network to regulate the synthesis, folding, translocation, and clearance of proteins. This network is challenged by the chronic expression of misfolded proteins in diseases of protein conformation, and by exposure to environmental and physiological stress. We have addressed this by establishing C. elegans models for expression of aggregation-prone proteins. Expression of Huntingtin polyQ proteins or mutant SOD1 in C. elegans muscle and neurons results in aggregation toxicity that is further amplified by expressed polymorphisms. As proteostasis is further destabilized during early adulthood of aging animals, this disrupts signaling pathways, causing cellular dysfunction and organismal failure. The collapse of proteostasis coincides with a dampening of the heat shock response (HSR) during aging. Activation of Hsf1 during early development, however, can suppress misfolding and enhance lifespan. Hsf1 is essential for the insulin-signaling pathway and is regulated by the NAD-dependent sirtuin, SIRT1. Stimulation of SIRT1 maintains Hsf1 in an active functional state during ageing of enhanced proteostasis. at the organismal level, the HSR in C. elegans is regulated by the AFD thermosensory neurons that sense temperature to regulate the expression of HS genes in somatic tissues by cell non-autonomous control. These results reveal that the HS response is organized at the systems level of the organism to sense the stress signal through active neuronal activity, and together with the metabolic state, sets the proteostasis network to ensure stability of the proteome and the health of the organism.

Minisymposium 23: The Nuclear Envelope and Nuclear Pore Complex (1609 -1614)

1609
The Permeability Barrier of Nuclear Pore Complexes.
The permeability barrier of nuclear pore complexes (NPCs) is a passive and yet highly efficient sorting device that controls all exchange between nucleus and cytoplasm. It suppresses the flux of inert macromolecules > 30 kDa, but also allows rapid passage of even very large cargoes, provided these are bound to appropriate nuclear transport receptors (NTRs). FG-repeat domains are the crucial elements of NPC function. They are essential for viability and comprise up to 50 repeat units. Each unit contains a hydrophobic cluster, typically of the sequence FG, FxFG or GLFG, which is surrounded by a more hydrophilic spacer sequence. FG repeats bind NTRs during facilitated NPC passage. Mutant NTRs that are defective in FG repeat binding also display defects in facilitated NPC passage. We observed that FG-repeats form hydrogels, which can be seen as reversibly crosslinked, 3-dimensional protein meshworks. These FG-hydrogels are fascinating materials that display permeability properties very similar to those of authentic NPCs, allowing an up to 20 000-fold faster entry of large NTR-cargo complexes compared to the cargoes alone. While supporting massive importin- or exportin-mediated cargo influx, such gels remain firm barriers towards inert objects that lack nuclear transport signals. This indicates that the FG-hydrogel reseals immediately behind a translocating species and thus possesses "self-healing" properties. The lecture will further address two key questions: what is the molecular and structural basis of barrier formation? And why do the meshes of the barrier open at least 100 times faster in the immediate vicinity of a nuclear transport receptor than elsewhere in the gel?

1610 Nuclear Pore Components Play an Essential Role in Developmental Transcription.
M. Capelson, Y. Liang, M. W. Hetzer; MCBL-Z, The Salk Institute for Biological Studies, La Jolla, CA

Over the last decades the analysis of nuclear pore complexes (NPCs) has focused on their role in nucleocyttoplasmic transport across the nuclear envelope (NE). More recently, NPCs have been implicated in gene activation and chromatin organization at the nuclear periphery in yeast, but their involvement in metazoan gene regulation remains unclear. Here we show that the nucleoporins Sec13, Nup98 and Nup88, as well as a group of FG-repeat nucleoporins, bind to the Drosophila genome at functionally distinct loci that often do not represent NE contact sites. While Nup88 localizes to silent loci, Sec13 and Nup98 bind to developmentally regulated genes undergoing induction of transcription. A subset of FG-repeat nucleoporins also bind to active genes and participate in a distinct induction step that is sensitive to inhibition of transcriptional elongation. Strikingly, RNAi-mediated knockdown of intranuclear Sec13 and Nup98 specifically inhibits transcription of their target genes without affecting general RNA polymerase II activity. Furthermore, Sec13 and Nup98 are required for efficient reactivation of transcription after heat shock, suggesting a novel essential role of NPC components in regulating complex gene expression programs of multicellular organisms.

1611 Transportin Regulates Major Mitotic Assembly Events: from Spindle Assembly to Nuclear Pore Assembly.
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Mitosis in higher eukaryotes is marked by the sequential assembly of two massive structures, the mitotic spindle and nucleus. for the latter, this further requires the precise formation of both nuclear membranes and nuclear pore complexes (NPC). Previously, importin alpha Beta and RanGTP were shown to act as dueling regulators to ensure that these processes, from
spindle assembly to nuclear pore assembly, occur only in the vicinity of the mitotic chromosomes. We now find that the distantly related karyopherin, transportin, is a cell cycle regulator. Transportin negatively regulates both nuclear membrane fusion and nuclear pore assembly in Xenopus egg extracts. For pore assembly, we show that these karyopherins initiate their regulation as early as the first known step, recruitment of the critical pore-targeting nucleoporin ELYS/MEL-28 to chromatin. Indeed, both transportin and importin beta can interact directly with ELYS. We further define the nucleoporin subunit targets for transportin and importin beta and find them to be largely the same: ELYS, the Nup107/160 complex, Nup53, and the FG nucleoporins. Equally importantly, we find that transportin negatively regulates mitotic spindle assembly. All of the above negative regulatory events are counteracted by RanGTP. We conclude that the interplay of the two negative regulators, transportin and importin beta, along with that of the positive regulator RanGTP, allows a precise choreography of multiple major cell cycle assembly events.

1612
Overlapping Functions of Nuclear Envelope Proteins NET25 (Lem2) and Emerin in Regulation of ERK Signaling in Myoblast Differentiation.
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Mutations in certain nuclear envelope (NE) proteins cause muscular dystrophies and other disorders, but the disease mechanisms remain unclear. The Nuclear Envelope Transmembrane protein NET25 (also referred to as Lem2) is a truncated paralog of MAN1, a NE component that is linked to bone disorders and known to regulate TGF-β/SMAD signaling. NET25 and MAN1 share a ~40 residue LEM homology domain with emerin, the protein mutated in X-linked Emery-Dreifuss muscular dystrophy and a regulator of MAPK signaling. However, roles for NET25 and MAN1 in myogenesis have not yet been described, and a signaling function of NET25 has not been demonstrated before. Using RNAi in C2C12 myoblasts, we show for the first time that both NET25 and MAN1 are required for myogenic differentiation. Ectopic expression of silencing-resistant NET25 rescues myogenesis after depletion of emerin but not after MAN1 silencing. Thus NET25 and emerin might interact genetically and have at least partially overlapping functions during myogenic differentiation distinct from those of MAN1. NET25 depletion causes hyper-activation of ERK1/2 at the onset of myoblast differentiation, and pharmacological inhibition of this transient overactivation rescues myogenesis. Results from our ongoing NET25 protein interaction study should reveal mechanistic details of how NET25 regulates ERK signaling, and might provide new clues as to how the nuclear envelope in general functions as a cell signaling platform. In summary, our work supports the hypothesis that deregulation of cell signaling contributes to NE-linked disorders. It also suggests that mutations in NET25 and MAN1 may cause muscle diseases, a possibility we are currently testing using an animal model.

1613
dll Spectrin is As an Essential Structural and Mechanical Component of Nucleoskeleton.
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The nucleoskeleton, also known as the nuclear lamina network, is primarily composed of A-type and B-type lamins which are type V intermediate filament proteins. A cortical network of lamin filaments alone, which behave mechanically as stiff rods, is insufficient to explain all measured properties of nuclei, including the ability to spring back after mechanical stretch. We hypothesize that the structure and mechanical resilience of the cell nucleus, like the cytoplasm, is determined by interconnections of multiple structural elements. We have visualized endogenous dll-spectrin and actin enriched at, but not limited to, the nuclear envelope and discovered that cells downregulated for dll-spectrin have major nuclear structural phenotypes. HeLa cells with reduced dll-spectrin have large, outwardly-dilated nuclei with significant lamin network reorganization and dilation. These nuclei, when challenged to stretch with a micropipette, deform but fail to "spring back" after mechanical stretch. Such phenotypes are distinct from LMNA-deficient nuclei,
suggested αII-spectrin contributes uniquely as a scaffold and spring in the nucleoskeleton. Thus, while not a load bearing element of the nucleoskeleton, αII-spectrin is an essential structural element which is important for both lamina stabilization and recovery of nucleus.

1614
Nuclear Transport Factors: From Viral Pathogenesis to Cell Division.
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Nuclear export of mRNAs is a central step in eukaryotic gene expression and it is highly regulated by viruses and interferons (IFN). Viruses subvert host cell mechanisms to favor their replication and in the process reveal key steps in host pathways that are critical points for regulating antiviral responses and which might be targets for therapeutic intervention. Influenza virus through its NS1 protein and VSV through its M protein each inhibit mRNA nuclear export, down-regulating host gene expression and antiviral responses. Based on the knowledge of NS1 functions in the nucleus as an inhibitor of gene expression, we performed a high throughput screen (HTS) of 200,000 small molecules. We identified novel compounds that significantly restored mRNA export in the presence of NS1 or infection and that significantly inhibited influenza virus replication and cytotoxicity. Among the most potent compounds was a member of the napthalimide family that targeted specific host pathways involved in antiviral response and nucleocytoplasmic trafficking. Certain constituents of the mRNA export machinery, which regulate antiviral response in interphase, acquire new functions in mitosis, such as key members of the Nup107-160 complex. We report here the interaction of the Nup107-160 complex, which is localized at the mitotic spindles and kinetochores, with an active form of the γ-tubulin ring complex (γ-TuRC), an essential and conserved microtubule nucleator. We showed that γ-TuRC localized to unattached mitotic kinetochores in a manner that requires the Nup107-160 complex, which coordinately promoted nucleation of microtubules near mitotic chromosomes and at kinetochores. Microtubule nucleation mediated by γ-TuRC and the Nup107-160 complex was regulated by RanGTP. Thus, our findings indicate that the Nup107-160 complex promotes spindle assembly through Ran-GTP regulated nucleation of microtubules by γ-TuRC at kinetochores.

Symposium 5 - Cellular Sociology: Working Together in Morphogenesis (1615 – 1617)

1615
Building the Vertebrate Spine.
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The vertebrate body can be subdivided along the antero-posterior (AP) axis into repeated structures called segments. This periodic pattern is established during embryogenesis by the somitogenesis process. Somites are generated in a rhythmic fashion from the paraxial mesoderm and subsequently differentiate to give rise to the vertebrae and skeletal muscles of the body. Somite formation involves an oscillator-the segmentation clock-whose periodic signal is converted into the periodic array of somite boundaries. This clock drives the dynamic expression of cyclic genes in the presomitic mesoderm. Microarray studies of the mouse presomitic mesoderm transcriptome revealed that the segmentation clock drives the periodic expression of a large network of cyclic genes involved in cell signaling. Mutually exclusive activation of the Notch/FGF and Wnt pathways during each cycle suggests that coordinated regulation of these three pathways underlies the clock oscillator. In humans, mutations in the genes associated to the function of this oscillator such as Dll3 or Lunatic Fringe result in abnormal segmentation of the vertebral column such as those seen in congenital scoliosis. Whereas the segmentation clock is
thought to set the pace of vertebrate segmentation, the translation of this pulsation into the reiterated arrangement of segment boundaries along the AP axis involves dynamic gradients of FGF and Wnt signaling. The FGF signaling gradient is established based on an unusual mechanism involving mRNA decay which provides an efficient means to couple the spatio-temporal activation of segmentation to the posterior elongation of the embryo. Another striking aspect of somite production is the strict bilateral symmetry of the process. Retinoic acid was shown to control aspects of this coordination by buffering destabilizing effects from the embryonic left-right machinery. Therefore somite development provides an outstanding paradigm to study patterning and differentiation in vertebrate embryos.

1616
**Cell Flow Reorients the Axis of Planar Polarity in the Wing Epithelium of Drosophila.**
B. Aigouy¹, R. Farhadifar², D. Staple², A. Sagner², J. Roeper², F. Jülicher², S. Eaton¹; ¹Eaton Lab, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, ²Max Planck Institute for the Physics of Complex Systems, Dresden, Germany

Planar cell polarity (PCP) proteins form polarized cortical domains that govern polarity of external structures such as hairs and cilia in both vertebrate and invertebrate epithelia. The mechanisms that globally organize PCP domain polarity are not understood. We investigate this problem in the Drosophila wing, using a combination of experiment and theory. Here we show that tissue remodelling is responsible for globally aligning PCP domains with the proximal-distal axis of the wing. We find that PCP domains are initially oriented towards the well-characterized organizer regions that control growth and patterning. At pupal stages, the wing hinge contracts and wing blade epithelial cells are stretched along the proximal-distal axis. In this process, precise patterns of tissue flow elongate the blade proximo-distally. These same tissue rearrangements realign planar polarity with the proximal-distal axis. This novel mechanism utilizes the cellular movements that sculpt tissues to align planar polarity with tissue shape.

1617
**Cellular and Molecular Dissection of Mammalian Branching Morphogenesis.**
M. Krasnow; Department of Biochemistry, Stanford University School of Medicine/HHMI, Stanford, CA

Many of our major organs consist of tubular networks that form by branching morphogenesis. Work in tractable genetic systems like Drosophila and C. elegans have begun to reveal the cell dynamics and molecular pathways that underlie branching morphogenesis, but understanding of the process for in the much more complex mammalian organs remains rudimentary. We will describe the development and application of methods for marking and following the fate of individual cells during branching morphogenesis of the lungs and pulmonary and coronary arteries in mouse, and how the results have overturned current models for how these structures arise during development. We will also describe the systematic genetic and genomic approaches we are using to elucidate the molecular pathways controlling these processes, and how they are altered in disease.

**Symposium 6 - Movers and Shapers: Nuclear Dynamics and Gene Regulation (1618 – 1620)**

1618
**Reorganization of Nuclear Lamina - Genome Interactions during Differentiation.**
B. van Steensel; Division of Gene Regulation, Netherlands Cancer Institute, Amsterdam, Netherlands

The three-dimensional organization of chromosomes within the nucleus and its dynamics during differentiation are largely unknown. The nuclear lamina is thought to provide an important
anchoring scaffold for the organization of interphase chromosomes, and may also play an active role in the regulation of gene expression. We present a high resolution genome-wide analysis of the interactions between chromatin and the nuclear lamina during differentiation. We mapped these interactions in mouse embryonic stem cells, lineage-committed neural precursor cells, and terminally differentiated astrocytes that are sequentially derived from one another. Chromatin in each of these cell types shows a similar organization into large lamina associated domains (LADs), which represent a transcriptionally repressive environment. During sequential differentiation steps, lamina interactions are progressively modified at hundreds of genomic locations. This remodeling involves both individual transcription units and multi-gene regions, and affects many genes that determine cellular identity. Often, genes that move away from the lamina are concomitantly activated; many others however remain inactive yet become unlocked for activation in a next differentiation step. These results suggest that lamina-genome interactions are widely involved in the control of gene expression programs during lineage commitment and terminal differentiation.

1619
Live Cell Dynamics of DNA Damage Response and Transcription.
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Endogenous and environmental agents continuously damage DNA causing transcriptional interference and disturbed cellular homeostasis. Lesion-induced replication errors create genomic instability, leading to malignant transformations. DNA damage response (DDR) mechanisms, including diverse DNA repair pathways protect organisms against the adverse effects of genomic insults. Inherited defects in DDR are associated with severe diseases, including progeroid and extreme cancer-prone syndromes. Nucleotide Excision Repair (NER) is a versatile DNA repair process able to remove different lesions. NER is intimately linked to transcription, illustrated by the pivotal role of TFIIH in both processes. Mutations in TFIIH are associated with a perplexing clinical heterogeneity, ranging from highly cancer-prone symptoms to premature-aging features. Despite detailed knowledge of each of the separate mechanisms transcription and NER, little is known about the dynamic interplay and regulation between these processes and how different mutations in the same protein complex can cause such different clinical outcomes. Protein dynamics and reaction kinetics of NER and transcription were determined by live cell imaging and photobleaching on cells expressing GFP-tagged factors and revealed a highly dynamic ‘on the spot assembly’ model of both processes. However, these studies were performed in cultured cells. It is unknown to which extent this highly dynamic organization holds for somatic tissues with differentiated and post-mitotic cells. To allow In Vivo analysis of transcription and repair in living tissues, we created a mouse-model that expresses YFP tagged XPB (subunit of TFIIH) from the endogenous gene locus. We determined NER and transcription kinetics in post-mitotic and highly differentiated cells embedded within their natural environment. Moreover, genetic crosses with existing TFIIH mutant mouse-models allows the analysis of the fate of mutated TFIIH and its effect on repair and transcription in relevant (affected) tissue. A surprising difference in both the steady-state level and the distribution over distinct kinetic pools of TFIIH between different cell types was observed in living tissue.

1620
The Nuclear Lamins: Building Blocks of Nuclear Architecture.
R. Goldman1, S. A. Adam1, A. Goldman1, T. Dechat1, P. Taimen1, T. Shimi1, K. Pfleghaar2;  1Feinberg School of Medicine, Northwestern University, Chicago, IL,  2Ludwig-Maximilians University, Munich, Germany

The nuclear lamins are Type V intermediate filament proteins. The A- and B-type lamins form separate nuclear networks that are concentrated in the nuclear lamina and are also present in lesser amounts throughout the nucleoplasm. The lamins are involved in many functions including
DNA replication, mitosis, transcription, the organization of interphase chromosome territories and in the overall shape and mechanical properties of nuclei. Remarkably, more than 300 mutations have been reported in the gene encoding human nuclear lamin A. These mutations cause numerous diseases including cardiomyopathies, lipodystrophies, muscular dystrophies and the premature aging disease, Progeria. Pathological hallmarks of these diseases include blebbed or lobulated nuclei, the mislocalization of nuclear pores and membrane proteins, and significant alterations in the epigenetic regulation and organization of chromosomes. However, little is known about the specific cellular and molecular mechanisms altered by the expression of mutant lamin A in patients with “laminopathies”. Therefore, we and our collaborators have developed methods for analyzing the impact of the mutant proteins on normal nuclear functions, ranging from live cell imaging to In Vitro lamin assembly assays and the microdissection and analysis of DNA in subnuclear regions of cells derived from patients. The data obtained to date are shedding new light on normal lamin functions, especially with regard to the roles of different domains in the ILamin A protein and disease-related dysfunctions of both the A and B-type lamins. Our results have led us to hypothesize that lamins form a nuclear scaffolding complex that provides a molecular interface between chromatin and the lamina/nuclear envelope complex and between chromatin and other components of the nucleoplasm. This lamin scaffold also acts as a platform for the assembly of protein complexes involved in a wide range of functions. Supported by the NIA, NCI and the Ellison Medical Research Foundation.

E.B. Wilson Awardee (1621)

1621
Protein Homeostasis in Health and Disease.
P. Walter; Biochemistry&Biophysics, HHMI/University of California, San Francisco School of Medicine, San Francisco, CA

Proteins are the building blocks of cells that carry out most essential functions, including cell-cell communication. As such, it is of central importance that they transmit information with fidelity, or a cell may receive improper signals and respond inappropriately. For any multicellular organism such miscommunication can be fatal, as it can lead to inappropriate cell death, uncontrolled cell division, or aberrant differentiation. Eukaryotic cells have evolved elaborate mechanisms by which they facilitate and control protein folding, and shunt misfolded proteins into degradative pathways before they can do harm. A particularly sophisticated mechanism resides in the endoplasmic reticulum (ER), the organelle in which secretory and membrane proteins begin their journey to the cell surface or extracellular space. Before leaving the ER, proteins are quality-controlled, and only properly folded proteins are allowed onwards. An "unfolded protein response" or UPR detects an insufficiency in the protein folding capacity in the ER and adjusts the abundance of the organelle according to need. The molecular machines that transmit this information and activate appropriate gene expression programs are simple in their molecular composition yet use unusual, unprecedented ways to carry out their functions, rendering the UPR a rich field for discovery of new signal transduction mechanisms. Important for a multicellular organism, if a protein folding defect in the ER cannot be corrected, the UPR switches from a cell protective mode to a cell destructive mode and activates apoptosis. The central role of the pathway in making life/death decisions connects the UPR to numerous diseases, including viral infection, protein folding diseases, diabetes, and cancer. Thus it is possible that newly described mechanistic insights can eventually be exploited therapeutically in new ways to attack human disease.

Cancer III (1622 – 1653)

1622/B1
Cleavage Failure Is Not a Strong Driver of Centrosome Amplification in Populations of Transformed Cells.
Cleavage failure has been proposed to be a leading event in the establishment of centrosome amplification (CA) in cells of human preneoplastic lesions and tumors. We previously reported that repeated cycles of experimentally induced cleavage failure do not establish centrosome amplification in populations of normal human cells. When tetraploid, these cells progressively drop out of the cell cycle and none divide more than three times. Thus, we test if cleavage failure as a transient event can establish centrosome amplification in populations of transformed cells with a defective p53 pathway. After cytochalasin induced cleavage failure, all HeLa cells die or arrest in interphase within 4 cell cycles while the same preparation diploid cells proliferate at ~100% frequency. HCT116 p53-/- cells, after cleavage failure, either arrest in interphase or start to repeatedly fail cleavage leading to huge cells that eventually die. Either way the cells do not proliferate after cleavage failure. CHO cells tolerate tetraploidy and proliferate after cleavage failure. In population studies we find that five rounds of cytochalasin induced cleavage failure at 5 day intervals raise the incidence of CA to 5% from 3% in the control cultures. Interestingly, the incidence of CA is stable and does not increase with more rounds of cleavage failure. Also, the incidence of CA is similar in the short (100 hour) and long terms (7 passages). We conclude that: 1. Cleavage failure as a transient event is not a strong driver of CA in populations of transformed cells, even those that proliferate as tetraploids. 2. The response of transformed cells to cleavage failure is highly variable and cell type specific. 3. Experimental results obtained with any particular transformed cell line may not be universally applicable to normal cells or other transformed cells.

1623/B2
Insights into the Warburg Effect: Aldolase Knockdown Inhibits the Growth of Ras-Transformed NIH-3T3 Cells.
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In cancer cells, a major metabolic shift occurs. The rate of glycolysis increases, while oxidative phosphorylation decreases, even in the presence of oxygen. This observation was first made in the 1950's by Otto Warburg, and is therefore known as the “Warburg Effect.” Because of this shift, glycolytic enzymes could be targets for interfering with the survival of cancer cells. Recently, several independent studies have tested this hypothesis for the glycolytic enzymes phosphofructokinase, lactate dehydrogenase, phosphoglucose isomerase, and pyruvate kinase using RNAi. Modest and variable effects on growth rate, glycolytic flux, and metabolite levels in an assortment of cancer cell lines were observed. In this study, other glycolytic enzymes, including aldolase, were knocked down using RNAi to determine the effect of these enzymes on cell growth rate in both untransformed NIH-3T3 cells and Ras-transformed 3T3 cells. Aldolase depletion revealed a dramatic effect on growth rate for both cell types. When aldolase a is knocked down in Ras-3T3 cells (85%), culture growth is completely inhibited for up to four days post-transfection, while cell viability shows a slight decrease. In contrast, similar knockdown of aldolase in normal NIH-3T3 cells (75%) shows a 70% decrease in growth rate, and cell viability is unaffected. Glycolytic flux rates and intracellular ATP levels are not affected by aldolase knockdown in normal NIH-3T3 cells, and these parameters will also be tested for Ras-3T3 cells.

1624/B3
Regulation of Cyclooxygenase-2 by Rhus Verniciflua Stokes in AGS Gastric Cancer Cells.
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Objective: Regulation of COX-2 enzyme, overexpressed in several human cancer cells, is an important strategic target in cancer therapy. Moreover, the expression of COX-2 in human gastric adenocarcinoma tissues which contained significantly higher levels of COX-2 mRNA when compared with paired gastric mucosal specimens except for cancer cells. Here, we evaluated that RVS extract, and its EtOAC fraction had the inhibition effect of COX-2 expression in gastric
cancer cells. Methods: 1) AGS cancer cell lines were treated with RVS extract, and its EtOAC fraction and Western blotting was performed to determine whether the observed differences in amounts of COX-2. 2) Which the mechanism by which RVS reduces the expression of COX-2 was evaluated. Results: 1) The reduction of COX-2 expression by an ethanol extract of Rhus verniciflua Stokes (RVS), purified phenolic-rich EtOAC fraction (PEF) from Rhus verniciflua Stokes ethanol extract were not mediated by transcriptional regulation. 2) Inhibition of COX-2 expression by an ethanol extract of Rhus verniciflua Stokes (RVS) and purified phenolic-rich EtOAC fraction (PEF) from Rhus verniciflua Stokes ethanol extract via ubiquitin-proteasome degradation. 3) The PKC-Raf-1-ERKMAPK signaling pathway is involved in COX-2 mRNA stability. 4) 3 common compounds-butein, sulfur ethin, and protocatechuic acid (the PEF from the RVS ethanol extract and RVS ethanol extract contained these compounds) had no effects COX-2 down-regulation. 5) Cell growth inhibition by an ethanol extract of Rhus verniciflua Stokes (RVS), phenolic-rich EtOAC fraction (PEF) from Rhus verniciflua Stokes ethanol extract was mediated COX-2 knock-down. These results suggest that RVS extract and fraction, the PEF, appears to regulate COX-2 expression by ubiquitin-proteasome degradation and by posttranscriptional mechanism (mRNA stability) via ERKMAPK inactivation in gastric AGS cancer cells. And RVS extract and fraction, the PEF, induced cell growth inhibition. Conclusions: Therefore, it is concluded that this medicine could be a targeted regulation of COX-2 molecule through different two mechanisms.

1625/B4
Inhibition of Epidermal Growth Factor Signaling by Cardiac Glycosides.
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Epidermal growth factor (EGF) signaling is important in regulating cell growth, proliferation, and differentiation. Upon binding to its receptor, EGF triggers cascades of downstream signaling, including activation of MAPK, PI-3 kinase (PI3K)/Akt, Jak/Stat, and protein kinase C pathways. Aberrant expression/activation of EGFR is found in multiple human cancers and is associated with metastasis, poor prognosis and resistance to chemotherapy. Inhibition of EGFR signaling can impair tumor growth and, thus, EGFR is an attractive target for cancer therapy. Therapeutic agents developed so far include antibodies against the ligand-binding domain and small molecules that inhibit EGFR's tyrosine kinase activity. Although these approaches have advanced cancer treatment, the response rate of 10-20% remains low. New approaches to inhibit EGFR are needed to improve therapeutic benefits to cancer patients. Cardiac glycosides are steroid-like compounds that inhibit Na,K-ATPase, a well-studied ion pump. In addition, recent studies revealed an additional role for Na,K-ATPase as a signal transducer, including MAPK and PI3K signaling. While both EGFR and Na,K-ATPase modulate similar signaling pathways, cardiac glycosides exhibit in contrast to EGFR anti-proliferative and pro-apoptotic properties in cancer cells. In this study, we sought to better understand the relationship between EGF and cardiac glycoside signaling in cancer cells derived from medulloblastoma, a predominant from of brain cancer in children. We show that in medulloblastoma cells, both EGF and the cardiac glycoside ouabain activated Erk1/2 and PI3K/Akt signaling. However, ouabain did not transactivate EGFR as reported earlier in other cell lines. Indeed, ouabain inhibited EGF-induced activation of Erk1/2 and Akt without affecting EGFR phosphorylation and abolished EGF-induced formation of stress fibers. Thus, these studies provide evidence that ouabain induced signaling can inhibit EGFR downstream signaling although similar pathways are activated when EGF or ouabain are present alone. These studies further suggest that ouabain activates additional pathways that are integrated to inhibit EGF-induced signaling in cancer cells.

1626/B5
Investigating the Oncogenic Potential of the Dual Specificity Phosphatase 12.
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Dual specificity phosphatases (DUSPs) are the predominant negative regulators of mitogen-activated protein kinase (MAPK) signaling pathways. DUSPs are an increasingly attractive target for drug therapies as MAPK signaling pathways are implicated in a variety of diseases including cancer. DUSP12 is one of only two candidate genes for the target of a 1q23 amplification found in highly invasive liposarcomas, however very little is known about DUSP12. The objective of this study is to investigate the function of DUSP12 and evaluate its oncogenic potential. We established a human cell line over-expressing a green fluorescence protein (GFP) DUSP12 fusion protein, and initial studies reveal faster wound closure as demonstrated by a scratch wound assay, and an increased proliferation compared to a GFP expressing control cell line. Interestingly, we found that over-expression of DUSP12 does not impact MAPK signaling in response to a variety of stimuli. In addition, using purified DUSP12, we have demonstrated that DUSP12 has low phosphatase activity in vitro. Further insights into the function of DUSP12 can be found in studies of its Saccharomyces cerevisiae homolog, yeast vaccinia homolog 1 (YVH1), that regulates cell growth and sporulation. Human DUSP12 can functionally substitute YVH1 in yvh1 knock out strains, indicating an evolutionarily conserved role of DUSP12 from yeast to humans. Intriguingly, we have found that YVH1 and DUSP12 regulation of cell growth in yeast is dependent not on its catalytic phosphatase domain, but on its highly conserved C-terminal domain of unknown function. In conclusion, our data suggests that DUSP12 is an evolutionarily conserved protein that regulates growth not by modulating MAPK signaling, but through the evolutionarily conserved C-terminal domain whose function we are currently investigating.

1627/B6
Cytotoxic Effects of Dehydroeburicoic Acid in Human U87MG Glioblastomas.
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The crude extract of Antrodia Camphorata has been reported to display cytotoxic effects in various tumor cell lines. It remains unclear, however, what compounds within the crude extract are responsible for generating the cytotoxic effect. We therefore examined the anti-proliferation effect of 15 kinds of triterpenoids purified from Antrodia Camphorate on human U87MG glioblastoma, one of the most frequent and malignant brain tumors in adults. MTT assay indicated that one of the triterpenoids purified from Antrodia Camphorat, dehydroeburicoic acid (DeEA), effectively inhibited cell proliferation. We aim to test the hypothesis that DeEA possesses significant cytotoxic effect against glioblastomas. LDH release assays indicated that DeEA enhanced LDH release in a concentration- and time-dependent manner in U87MG cells. In addition, Annexin V and propidium iodide staining showed that DeEA treatment led to an increased presence of glioblastomas in the necrotic/late apoptotic fraction, whereas cell cycle analysis revealed that DeEA failed to significantly enhance the population of U87MG cells in the hypodiploid (sub-G1) fraction. Using electron microscopy, we found that DeEA induced significant cell enlargements, massive cytoplasmic vacuolization, and loss of mitochondrial membrane integrity. Importantly, DeEA cytotoxicity in U87MG cells was caspase-independent. DeEA treatment also triggered an intracellular calcium increase, and DeEA-induced cell death was significantly attenuated by BAPTA-AM, but not EDTA or EGTA. DeEA induced a reduction of mitochondrial transmembrane potential. Moreover, we demonstrated that calpain inhibitors effectively blocked LDH release from DeEA-treated cells. Taken together, these results suggest that in human glioblastomas, DeEA induces necrotic cell death that involves calcium overload, mitochondrial dysfunction, and calpain activation.

1628/B7
Enhanced Cytotoxicity and Intravesical Drug Delivery of Epirubicin Nanoparticles.
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Objective: Epirubicin is a chemotherapy drug currently used in the intravesical instillation for superficial bladder cancer. The aim of this study was to develop poly(ethyl-2-cyanoacrylate) (PECA) epirubicin-loaded nanoparticles (EPI-NP). Methods: A 2^3 factorial design was adopted with the type of surfactant, surfactant concentration and the pH of the polymerization medium as independent variables. The particle size, entrapment efficacy and polydispersity index of eight formulations were then evaluated. Results: Two optimal EPI-NP formulations, 2% Tween 80 EPI-NP (TW80 EPI-NP) and 0.5% pluronic F68 EPI-NP (F68 EPI-NP) at pH 2.5 were developed. The sizes of TW80 EPI-NP and F68 EPI-NP at maximum intensity were 90 nm and 220 nm, respectively. Both TW80 EPI-NP and F68 EPI-NP showed potent cytotoxicity against human bladder cancer T24 and RT4 cells, compared with aqueous solutions of epirubicin (EPI-AQ). The effects of EPI-AQ, TW80 EPI-NP and F68 EPI-NP on the cell viability of T24 and RT4 were determined by MTS assay. The EPI-AQ, TW80 EPI-NP and F68 EPI-NP caused 50% T24 cell death at concentrations of 25.85, 1.19 and 1.52 μg/mL, respectively. EPI-AQ, TW80 EPI-NP and F68 EPI-NP caused 50% RT4 cell death at concentrations of 2.00, 1.20 and 1.30 μg/mL, respectively. The cytotoxicity of TW80 EPI-NP and F68 EPI-NP toward both T24 and RT4 cells significantly exceeded that of EPI-AQ (p<0.05). The penetration and accumulation of EPI-NPs in pig urothelium were studied by tissue concentration-depth profiles and fluorescence microscopy. The cumulative amounts of epirubicin following EPI-AQ, TW80 EPI-NP and F68 EPI-NP treatments were 842.48 ± 24.66 μg, 1314.66 ± 33.07 μg and 595.21 ± 24.16 μg, respectively. Conclusions: The current study showed the successful development of urothelium adhesive and penetrative PECA EPI-NPs. This has potential for the In Vivo application of epirubicin-loaded nanoparticles for intravesical instillation in bladder cancer therapy.

1629/B8
Semaphorin 3A Potentiates Metastatic Disease by Binding to Neuropilin-1 and Disrupting Vascular Barrier Function.
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We previously showed that Semaphorin 3A (SemA3A), a known inhibitor of axonal sprouting, inhibits VEGF-induced angiogenesis yet induces vascular permeability. We considered whether its expression in a variety of highly malignant tumor cells might contribute to their metastatic properties based on SemA3A’s capacity to disrupt vascular barrier function. To evaluate this, we knocked down SemA3A expression in CT26 (colon), PAN02 (pancreas), and B16-BL6 (skin) cells and examined their growth and invasive properties both In Vitro and in vivo. SemA3A knockdown did not impair tumor cell adhesion, migration, proliferation, or anchorage-independent growth In Vitro and had no effect or primary tumor growth in vivo. However, loss of SemA3A significantly prevented the capacity of these cells to form pulmonary lung metastases. Genetic disruption of the SemA3A receptor Neuropilin-1 (Nrp1) or expression of Nrp1 mutant lacking the SemA3A binding site within the adult endothelium stabilized vascular barrier function and inhibited lung colonization and metastasis. Together these findings define a pathological role for tumor cell secreted SemA3A as a potentiator of tumor cell metastasis due to its capacity to disrupt vascular barrier function.

1630/B9
The ERAD Inhibitor EerI Inhibits a p97-Associated Ubiquitin Processing Reaction to Induce Cancer Cell Death.
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The ubiquitin-proteasome system (UPS) has recently emerged as a major target for drug development in cancer therapy. The proteasome inhibitor bortezomib has cytotoxic activity in multiple myeloma and mantle cell lymphoma. Here we report that Eeyarestatin I (EerI), a chemical inhibitor that blocks ER-associated protein degradation (ERAD) via binding to a p97
complex, has anti-tumor and biologic activities similar to bortezomib, and can synergize with bortezomib. Like bortezomib, EerI-induced cytotoxicity requires the upregulation of the BH3 only pro-apoptotic protein NOXA. We further demonstrate that both EerI and bortezomib activate NOXA via a novel mechanism that requires cooperation between two processes: First, these agents elicit an integrated stress response program at the ER to activate the CREB/ATF transcription factors ATF3 and ATF4. We show that ATF3 and ATF4 form a complex capable of binding to the NOXA promoter, which is required for NOXA activation. Second, EerI and bortezomib also block ubiquitination of histone H2A to relieve its inhibition on NOXA transcription. Consistent with the notion that EerI targets a p97 complex, knock-down of p97 also elicits cellular responses similar to EerI treatment. Our results identify a class of anti-cancer agents that target the cytosolic AAA ATPase p97 to induce cell death.

1631/B10
Development of a GPCR-Blocking Monoclonal Antibody for the Treatment of Cancer.
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Sphingosine 1-phosphate (S1P) has been shown to mediate a variety of physiological and pathological processes, but its role in the tumorigenesis of breast cancer is incompletely characterized. Here we examined the effects of extracellular S1P on two breast cancer cell (BCC) types: MCF-7 and MDA-MB-231 cells. We found that S1P increased proliferation and tamoxifen resistance in both cell types, but induced cytoskeletal rearrangement and enhanced motility only in late-stage MDA-MB-231 cells. The effect of S1P on cytoskeletal rearrangement was dependant on Rho-kinase (ROCK) but not on Rac1. Furthermore, S1P signaling increased the expression of growth factor receptors EGFR and Her-2 and decreased the expression of tumor suppressor p53. Expression analysis and RNAi studies suggest that these responses are largely the result of the activation of cognate G protein-coupled receptor (GPCR), S1P3. Correspondingly, S1P3-blocking antiserum was able to completely inhibit S1P-mediated cell rounding in MDA-MB-231 cells, and sensitized them to tamoxifen-mediated cell death. We have since developed a monoclonal antibody that specifically recognize s an extracellular domain of S1P3 and exhibits functional blocking activity. To our knowledge, this represents a first-in-class blocking monoclonal antibody for a GPCR. The continued development of blocking antibodies for S1P receptors represents a new therapeutic modality for the treatment of a variety of disease states including cancer.

1632/B11
Hepatitis B Virus X (HBx) Impairs hBubR1 Function through HBxAP/Rsf-1.
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Hepatitis B virus X (HBx), encoded by HBV genome, deeply involves in the development of HBV-mediated liver cancer and is shown to increase genomic instability. We have reported that HBx binds hBubR1, a major component of the mitotic checkpoint (Oncogene. 27(24):3457-64, 2008). Here, we showed a notably weak HBx/hBubR1 interaction in vitro, suggesting that a strong HBx/hBubR1 interaction In Vivo require other protein(s). We found that depletion of HBx-interacting protein (HBxIP) by siRNA did not distort the HBx/hBubR1 interaction but rather enhanced it. In contrast, RNA interference against HBxAP (HBx-associated protein), also known as Rsf-1 (a subunit of chromatin remodeling factor complex), abolished the HBx/hBubR1 interaction in vivo. Reconstitution of HBxAP/Rsf-1 into the HBxAP depleted cells restored the HBx/hBubR1 interaction, confirming that HBxAP/Rsf-1 mediates the HBx/hBubR1 interaction in vivo. Intriguingly, these interactions mainly occur during mitosis at the centromeric region. A series of deletion mutant analysis revealed that two Kunitz domains of HBx and the Cdc20 binding domain of hBubR1 are essential for these interactions. Consequently, the truncated
mutant deleting Kunitz domain of HBx failed to bind hBubR1 and did not cause mitotic aberration. Moreover, depletion of HBxAP/Rsf-1 diminished the HBx-mediated mitotic aberrations. Together, our data show that the Rsf-1/HBxAP mediates the HBx/hBubR1 interaction. This provides a novel mechanism for a viral pathogen to induce genomic instability through mitotic checkpoint dysfunction in hepatocarcinogenesis.

1633/B12
The Endoplasmic Retained AGR2 Induces CDX2 Expression in Primary Intestinal Epithelial Cells.
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Background/Objective: The AGR2 amino acid sequence features a 19 kD protein, a signal peptide, and an ER retention signal (KTEL). AGR2’s specific function is unknown, but has been implicated in Xenopus glandular organ development, newt limb regeneration, and human adenocarcinoma growth. Normal mouse intestine express AGR2 in secretory cells (i.e. Paneth, goblet, and enteroendocrine cells); including replicating cells within the intestinal crypt. Using a primary intestinal cell line, we explored potential mediators of AGR2 effects and the importance of its ER retention signal. Methods: IEC-6 cells originally isolated from rat intestinal crypts do not express AGR2. AGR2’s effect on intestinal cells was studied by stably transfecting AGR2 cDNA into IEC-6 cells. AGR2’s impact was evaluated using qPCR, immunoblotting, and immunofluorescence. We also studied the importance of AGR2’s carboxy-terminal KTEL sequence by introducing a stop before KTEL or conversion to a known ER retention signal, KDEL. Results: CDX2 expression is not detected in wild-type IEC-6 cells. qPCR revealed that AGR2 expression in IEC-6 cells induced CDX2 expression. CDX2 expression was confirmed with immunoblotting and immunofluorescence. CDX2 is a transcription factor that promotes intestinal development, including proliferation and differentiation. IEC-6 cells also exhibited enhanced cell proliferation with AGR2 expression. Using CDX2 expression as readout for AGR2, the carboxy-terminal KTEL sequence’s role was explored. CDX2 expression persisted after the AGR2 carboxy-terminal sequence was changed from KTEL to KDEL, which results in full ER retention. Insertion of a stop sequence prior to KTEL did not induce CDX2 expression. Conclusion: AGR2 induces CDX2 expression in IEC-6 cells; consistent with their known expression in proliferating and differentiating intestinal cells. Consistent with AGR2's established role in Xenopus glandular development, it may serve a similar role in mammalian intestinal development. AGR2 activity requires an ER retention signal, and implicates the ER as the location for its effects.

1634/B13
Tumor Necrosis Factor [TNF] Antagonists for the Treatment of Rheumatoid Arthritis [RA] Inhibit the Production of Tumor Markers.
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Using TNF antagonists, we tried to evaluate the role of TNF on the mechanism of the elevation of the tumor markers. In the patients with active 20 RA patients with the elevation of many kinds of tumor markers [CEA,AFP CA19-9 CA125 etc] in the peripheral bloods, achieved by a significantly greater portion [maximum, over 90%], the drop of the levels of tumor markers. Conclusion: We first found that TNF antagonists treatment inhibited the elevation of the tumor markers. This specific effect by monoclonal antibody shows that TNF may play an important role on the mechanism of the production of tumor markers.

1635/B14
The Hepatitis B Virus Middle Surface Protein Does Not Require Ubiquitination for Entry into The Endoplasmic Reticulum Degradation Pathway.
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Hepatitis B virus (HBV) infects more than 2 billion people worldwide, and approximately 350 million of those are chronic infections. Since a strong epidemiologic relationship exists between HBV infection and hepatocellular carcinoma (HCC) development, several hypotheses have been proposed as potential mechanism for cancer development. Several groups have shown that HBV envelope protein mutants are frequently isolated from patients with HCC. These mutants accumulate in endoplasmic reticulum (ER), induce ER stress and lead to oxidative DNA damage and gene instability. We have used one of these HBV envelope proteins, the HBV middle surface protein (M) as a model to study how ER quality control monitors these foreign polymeric proteins and disposes of them through the ER-associated degradation (ERAD) pathway. Surprisingly, we found that unlike any known ERAD substrates, which require ubiquitination during the process of retrotranslocation from the ER into cytosol, a misfolded M protein, which carries triple cysteines substitutions by alanines in order to prevent the normal inter-chain disulfide bonds formation, can undergo an ubiquitination independent, calnexin independent, proteasome depended degradation pathway. In contrast, misfolded M protein ΔSigI/II, which lacks the first two transmembrane domains, is ubiquitinated by Sel1l-HRD1 complex, an ERAD associated E3 ubiquitin ligase complex. However, this process is not crucial for degradation of ΔSigI/II by proteasome. Therefore, using HBV M protein as a model, we report a new mechanism for ER quality control in recognition and disposal of misfolded viral proteins into cytoplasm to be degraded by proteasome.

1636/B15
What Is the Function of Serum Amyloid A (SAA) Protein on Progression of Glioblastomas Multiformes?
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Factors synthesized by tumor cells act in autocrine and/or in normal cells of the host, especially in cells of the immune system, resulting in tumor growth. Recently it was reported that some tumors express the acute phase protein serum amyloid a (SAA). This protein is synthesized mainly by the liver during the inflammatory process. So far, the relationship between SAA and the cancer is restricted to clinical studies that describe the SAA as a diagnostic marker. However, the existence of direct relationship of SAA with the proliferation or cell death has not yet been investigated. In this study our purpose was to assess the direct effect of SAA in human glioma cell lineages - T98G and A172 - with regard to proliferation and migration of these cells. We also investigated the ability of SAA to induce IL-8, M-CSF, TNF-α by these lineages and whether the production of them is related to cell proliferation. Finally, we evaluate the expression of the SAA1, SAA2 and SAA4, of matrix metalloproteinases (MMP-2 and MMP-9) and RECK genes in the presence and absence of interferon-gamma (INF-γ), an inducer of the expression of SAA. T98G and A172 were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% or 0.5% fetal bovine serum (SFB) and stimulated with 0.1μg/mL, 1μg/mL, 5μg/mL and 20μg/mL of SAA by 48hours. for proliferation, 3[H] thymidine incorporation was used. The migration test was carried out by scratch test, for the quantification of the expression of SAA1, SAA2 and SAA4, MMP-2, MMP-9 and RECK, the gliomas were treated with 100ng/mL of INF-γ, the RNA was extracted, converted to cDNA and quantified by Real Time PCR. The production of TNF-α, IL-8 and M-CSF was measured by ELISA. SAA stimulates the proliferation of gliomas, the migration of T98G and the production of cytokines in the tumor environment. TNF-α was produced in both gliomas in presence of SAA, while IL-8 was produced only in A172 and M-CSF in T98G. Gliomas express all of the SAA genes and this expression is significantly enhanced by INF-γ. This also increases the expression of MMPs and decrease of RECK. The results suggest a pro-tumor activity for SAA that may have important implications in the prognosis of the development of tumors.

1637/B16
Monitoring Tryptophan Metabolism in Human Lineages of Gliomas and Melanomas.
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In several malignant diseases, accelerated tryptophan (TRP) catabolism has been described. Lower TRP concentration and increased kynurenine (KYN)/TRP ratio are associated with more advanced stages of disease. In tumor patients, it has been claimed that TRP degradation may represent an intrinsic immune escape mechanism. Endogenous formation of IFN-g during host response against the tumor increases the expression of the enzyme that metabolizes TRP, indoleamine 2,3-dioxygenase (IDO). The expression of IDO has been shown for different human tumors and, in these cases, the administration of IDO inhibitors has been proposed in combination with usually antineoplastic treatment. Thus, the recognition of tumors that express IDO is desirable as well as the response of these cells to common IDO inducers, especially IFN-g. Here we determine the constitutive expression and activity of IDO in two gliomas (A172 and T98G) and five melanomas lineages and the effect of IFN-g on these cells. The possibility that these lineages contain a coexisting via of TRP metabolism, that is, the serotonergic pathway, was also evaluated. Cells were cultured in the presence or absence of IFN-g. mRNA expression of indo, tph, aanat and hiomt were monitored by RT-PCR. IDO activity was measured by the common assay in cell homogenates. The activity of IDO was expressed by the amount of KYN formed by HPLC. The amount of KYN in cultures was also determined. For gene expression, indo was expressed in A172 and melanomas only in the presence of IFN-g, while for T98G, the KYN pathway is expressed even in the absence of the stimulus. tph, aanat and hiomt were poorly expressed in all lineages. The amount of KYN was IFN-g dose dependent in all lineages. Among the cells studied we found three profiles: cells that present IDO activity only in the presence of IFN-g, cells that present small IDO activity and responds poorly to IFN-g and cells that are strongly responsive to IFN-g (more than 10-fold increase in KYN). Future analysis of the association of IDO expression with the viability of these different cell lineages in cocultures with immune cells will substantially improve our understanding about the KYN and serotonergic pathways in tumor progression.

1638/B17
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The Abl oncogenes encode activated non-receptor tyrosine kinases that cause cancer in mice and human. The Abl proteins include the retrovirally-transduced product of Abi, v-Abl, and the product of chromosomal translocations found in human leukemias Bcr-Abl and TEL-Abl. There is increasing evidence suggesting that the full transforming activity of Abl involves the activation of several signaling pathways including PI3K/AKT, RAS/MAP kinase, and JAK/STAT. Recently, it has been reported that the Glu17Lys mutation in the AKT1 PH domain is associated with multiple human malignancies and leukemia in mice. However, this mutation has not been identified in Abl-transformed cells. Because the PI3K/AKT pathway plays an important role in Abl-mediated transformation, we investigated the presence of the AKT1 E17K mutation in v-Abl-transformed cell clones using a single-strand conformation polymorphism (SSCP) assay and direct sequencing. AKT1 E17K mutation was detected in 2 (2.1%) of 94 specimens examined. To directly demonstrate the involvement of AKT1 E17K mutation in Abl-mediated tumorigenesis, bone marrow cells from mice were infected with bicistronic retroviruses encoding the p120 form of v-Abl and either GFP, wild-type or the mutant AKT1. Interestingly, we found that E17K mutant greatly increased the v-Abl transformation efficiency as compared with wild-type AKT1. In addition, we observed that ectopic expression of E17K mutant significantly increased the phosphorylation levels of proapoptotic protein BAD. This correlated with an increased protection from imatinib-induced apoptosis in abl transformants. Furthermore, we have addressed the relationship between AKT and Pim by determining if activated AKT can complement Pim kinase deficiency for v-Abl-mediated transformation. Our study demonstrated that AKT and Pim-1 exhibit feedback relationship in v-Abl-transformed cells.
**1639/B18**

**Activation of Tissue Transglutaminase-mediated Apoptotic Cell Death in Photodynamic Therapy of Cancer Cells.**

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Photodynamic therapy (PDT) is an emerging modality for treatment of a variety of neoplastic and pre-neoplastic diseases; however, mechanisms of intracellular signaling responsible for PDT-induced cell death are not fully elucidated. In this study, we investigated the new role of tissue transglutaminase (tTGase) in cell death induced in human gastric adenocarcinoma and human bladder carcinoma cells by PDT with a photosensitizer, DH-II-24, using an on-stage system. DH-II-24-mediated PDT induced apoptotic cell death and activation of tTGase. tTGase activation was inhibited by cystamine and tTGase siRNA, both of which also reversed the PDT-induced apoptotic cell death. PDT produced intracellular reactive oxygen species and caused a transient increase in intracellular Ca2+. Reactive oxygen species scavengers, Trolox and N-acetyl-L-cysteine, and the Ca2+ chelator, BAPTA-AM, inhibited tTGase activation and prevented the induction of apoptotic cell death by PDT. Furthermore, PDT activated caspase-3 and this activation was inhibited by Z-VAD-fmk, Trolox, BAPTA-AM, cystamine and tTGase siRNA. Together, these results demonstrate that DH-II-24-mediated PDT targets tTGase by elevating intracellular concentrations of ROS and Ca2+, resulting in apoptotic cell death via activation of caspase-3. Moreover, this study suggests tTGase as a possible therapeutic target for PDT treatment of cancer.

**1640/B19**

**Functional Embryomas as a Result of Embryonic Gene Re-Expression.**

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**1641/B20**

**Identification and Subcellular Distribution of a Multimeric Protein Complex Encompassing Hsp90a.**

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Hsp90 is one of the most abundant cellular chaperones and its expression is elevated in cancer cells. In addition to forming various cytosolic complexes with co-chaperones and client proteins, Hsp90α has been identified at the cell surface, facing the extracellular space. In this study we used chemical crosslinkers with spacer arm of various lengths in an attempt to stabilize the binding of Hsp90α with interactor proteins in human hepatoma HepG2 cells. The results show that the crosslinker EGS [ethylene glycol bis(succinimidy)succinate] promoted the formation of a ~240 kDa complex encompassing Hsp90α without Hsp70 or p60(Hop). Similar results were found in a number of cancer cell lines and in human PBMCs. Pharmacological inhibition of Hsp90 with 17-AAG or novobiocin and expression of an Hsp90α mutant defective for intrinsic ATPase activity did not prevent formation of the complex by EGS. Covalent association of Hsp90α dimers was confirmed by reciprocal immunoprecipitation experiments using epitope-tagged Hsp90 constructs. Treatment of HepG2 cells with the cell impermeable sulfo-derivative of EGS caused detectable accumulation of a proteinase K-sensitive 240-kDa complex at the cell surface. The actin cross-linking and scaffold protein filamin a is a known Hsp90 interactor and plays a key role in intracellular trafficking of signaling molecules. siRNA-mediated gene silencing of filamin a did not reduce EGS-dependent formation and sorting of the 240-kDa Hsp90-containing multimeric complex to the cell surface. These studies show that chemical crosslinking is a viable approach to stabilize Hsp90 interaction with binding partner(s), whose participation in the control of Hsp90α cellular functions is awaiting further analysis. Efforts are underway to identify and characterize the interaction partner(s) of Hsp90 by mass spectrometry.

1642/B21
Regulation of Cell Growth and Proliferation by the Tropomyosin Tm5NM1: A Novel Target for Anti-cancer Chemotherapeutics.
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The actin cytoskeleton is an important regulator of a variety of cellular functions including motility, adhesion, and proliferation. Changes in these processes are fundamental to cellular transformation making the actin cytoskeleton a long sought after chemotherapeutic target. Drugs developed to date have been unsuccessful due to their lack of specificity which ultimately causes unacceptable cardiac and respiratory toxicity. OBJECTIVE: to develop drugs which specifically target actin filament populations that are fundamental for tumour cell proliferation. We have previously shown that tropomyosin (Tm), an integral component of the actin cytoskeleton, defines functionally distinct populations of actin filaments. We have identified a specific Tm isoform common to all tumour cells which regulates cell proliferation and have designed a new class of compounds to target this filament population. SUMMARY OF RESULTS: The role of Tm5NM1, a ubiquitously expressed low molecular weight Tm isoform, was investigated in both overexpression and knockout cell systems. Elevated levels of Tm5NM1 in the rat neuroepithelial B35 cells accelerated cell proliferation and enhanced anchorage independent growth in soft agar assays. Conversely, cell proliferation in response to serum was significantly reduced in primary mouse embryo fibroblasts isolated from a Tm5NM1 knockout mouse. siRNA knockdown of Tm5NM1 in the neuroblastoma tumour cell line (SHEP) also resulted in decreased cell growth. We have developed a novel class of anti-Tm compounds that target Tm5NM1 containing filaments. Our lead compound, TR100, is effective against a panel of neuroblastoma and melanoma cell lines (average LC50 ~2-3 μM) and significantly reduced tumour growth in the murine B16/F10 melanoma model. In Vivo data from the drug treated animals also showed no evidence of cardiac damage as measured by blood Troponin I levels and no obvious hypertrophy.
as measured by intraventricular septum thickness. CONCLUSIONS: We have demonstrated for the first time a novel class of chemotherapeutic compounds which specifically target an actin filament population required for cell growth and proliferation. This has enormous implications for the treatment of a variety of cancers.

1643/B22
**Blockade of Myeloid Cell Trafficking by Inhibiting a PI3-Kinase γ-α4 Integrin Activation Pathway Stimulates Anti-tumor Immunity.**
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Accumulation of CD11b+Gr1+ myeloid-derived suppressor cells (MDSC) and associated factors (VEGF, IL-6, IL-10) induce immunosuppression during tumor growth and metastasis. This immunosuppressive microenvironment causes a deficiency in dendritic cell (DC) maturation as well as dysfunctional T cell-mediated anti-tumour immunity, favouring tumor immune escape. Recently, we determined that defects in PI3Ky-α4 integrin signaling in myeloid cells reduces tumor inflammation, angiogenesis and progression. As blockade of myeloid cell trafficking reduces expression of inflammatory factors in the tumor microenvironment, we hypothesized that blockade of PI3Ky-α4 pathway stimulates anti-tumor immunity. Lewis Lung Carcinoma (LLC) tumor cells were subcutaneously injected into WT, integrin α4Y991A (which bear a mutation in the cytoplasmic tail of integrin reducing integrin signaling) or PI3Ky-/- mice. In some experiments, tumor-bearing mice were treated with integrin α4β1 or PI3Ky inhibitors. Tumors were harvested 2 weeks later and immune cell infiltration in the tumor microenvironment was analyzed. Here we show that the inhibition of the PI3Ky-α4 integrin activation pathway modulates the tumor microenvironment by decreasing MDSC infiltration and down-regulating immunosuppressive cytokines (IL6, IL10 and VEGF). More interestingly, blocking this pathway increases the percentage of mature DC in tumors. To further understand the mechanisms underlying the reversion of immune suppression mediated by blockade of PI3Ky-α4 integrin, we evaluated factors that may favor T-cell activation. PI3Ky inhibitors, as well as the PI3Ky-/- and α4YA mutations, enhance expression of antitumor cytokines (IL12 and IFNy), CD8+ T cell infiltration and tumor cell killing mediated by T cells. In conclusion, suppression of PI3Ky or integrin α4 activity inhibits tumor inflammation, angiogenesis and tumor progression and also stimulates antitumor immunity. These results indicate that PI3Ky and integrin α4 are valuable targets for the design of novel cancer therapeutics.

1644/B23
**Upregulation of EpCAM Expression by Axl in Colorectal Carcinoma Cells.**
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The tumor-associated antigen EpCAM (epithelial cell-adhesion molecule) has been known to be principally expressed in various human cancers including in colorectal carcinoma, but the regulatory mechanism in the tumorigenicity of colorectal carcinoma remains to be unraveled. To identify the candidate genes that are able to regulate EpCAM expression and elucidate their regulatory role in the tumorigenicity of colorectal carcinoma, we determined the candidate target genes. Among the genes, Axl receptor tyrosine kinase increased the expression of EpCAM in MC38 colorectal carcinoma cells. Treatment with Axl-Ig suppressed the metastasis as well as the proliferation of colorectal carcinoma cells in a dose-dependent manner. These findings collectively suggest that Axl plays a critical role in the regulation of EpCAM-mediated tumorigenicity of colorectal carcinoma, and Axl might be a potential target for the novel colon carcinoma therapies.

1645/B24
**Apoptosis-related Changes in Response to Microtubule Poisons in APC-Deficient Cells.**
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The Adenomatous Polyposis Coli (APC) gene is mutated in the majority of colorectal cancers. This causes a reduction in microtubule stability. Based on this defect, clinically used microtubule poisons like taxol and vinorelbine could be potentially useful chemotherapeutic agents for colorectal cancer. Surprisingly, APC-deficient cells arrest in mitosis less efficiently and die less readily when treated with these reagents. To understand the underlying mechanism for this defect and to identify potential Achilles’ heels of APC-deficient cells, we aimed to (1) determine how microtubule poisons kill cells and (2) identify the role of APC in this process. We compared the transcriptional profile of apoptosis-related genes after prolonged mitotic arrest induced by taxol in cells before and after depletion of APC. We found that taxol-mediated mitotic arrest specifically induced transcription of a number of apoptosis-related genes and that this response differed in APC-deficient cells, with the most striking difference in the level of Bcl-2 message and protein. APC status also altered the phosphorylation of Bcl-2 in mitotically arrested cells. We also discovered that, unlike taxol and nocodazole, vinorelbine kills cells directly from interphase, independently of mitotic arrest. Using live cell imaging and FACS analysis we showed that loss of APC and p53 independently increase cell survival in vinorelbine-treated cells. We conclude that (1) taxol-mediated mitotic arrest specifically induces transcription of apoptosis-related genes that could mediate apoptosis in response to prolonged mitotic arrest; (2) APC depletion alters this response in a manner that is partially independent of changes in Wnt Beta-catenin signaling; (3) APC and p53 contribute to vinorelbine-induced cell death initiated from interphase. Identifying factors that provide protection specifically to APC-deficient cells may reveal potential targets for treating a common human tumour type.

1646/B25

GRP75 Is Required for Neuroblastoma Cell Differentiation and Is a Favorable Prognostic Marker of Neuroblastoma.

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Neuroblastoma (NB) is the most common extracranial solid tumor of children and is originated from primitive sympathetic neural precursors derived from neural crest. Most children have advanced or metastatic status of disease at diagnosed age. Some of these metastatic tumors can spontaneously regress or mature into a benign ganglioneuroma by undergoing differentiation or apoptosis. We attempted to explore the factors that involved in neuroblastoma differentiation as an indicator for prognostic implication. Glucose regulated protein (GRP75) was highly expressed in human neuroblastoma cell line SH-SY5Y cells after retinoic acid (RA)-induced differentiation. Knockdown of GRP75 significantly decreased neuronal differentiation of SH-SY5Y cells in response to RA. Immunohistochemical analyses of NB tumor tissues further revealed that positive GRP75 immunostaining was strongly correlated with differentiated histologies, mass-screened tumors, and early clinical stages but inversely correlated with MYCN amplification. These findings clearly showed that GRP75 is essential for neuroblastoma cell differentiation and is a favorable prognostic indicator of neuroblastoma.

1647/B26

Tks Proteins Regulate Localized ROS Formation by NADPH Oxidases and Promote Assembly of Invadopodia in Colon Cancer Cells.

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The NADPH oxidase (Nox) family, consisting of Nox1-5 and Duox1-2, catalyzes the regulated formation of reactive oxygen species (ROS). Members of this family have been implicated in many patho-physiological processes. The transmembrane protein Nox1 is highly expressed in the
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colon and requires for its full activity the cytosolic regulators NoxO1, NoxA1 and Rac1 GTPase. Although the regulation of Nox1-dependent ROS generation is still poorly understood, a number of Nox-mediated biological processes require compartmentalized ROS formation. The c-Src substrates, Tks4 and Tks5, contain an NH2-terminal Phox (PX) domain and four (Tks4) or five (Tks5) Src homology 3 (SH3) domains. Recently, the Courtneidge lab demonstrated that Tks5-dependent, Nox-mediated ROS generation is necessary for formation of invadopodia, cellular actin structures that degrade extracellular matrix and promote cell invasion. We show that Tks proteins are novel members of the p47phox organizer superfamily which selectively support Nox1 and Nox3 (vs. Nox2 and Nox4) activity by binding the NoxA1 activator protein via an SH3-mediated interaction. Human DLD1 colon cancer cells express endogenous Nox1, NoxA1 and Tks4, and their ability to generate both ROS and invadopodia is inhibited by the siRNA-mediated depletion of Tks4. Tks4 recruits Nox1 to invadopodia, where localized ROS production occurs to support formation of invadopodia. Intriguingly, the overexpression of a different p47phox-superfamily organizer subunit (NoxO1) strongly reduces the formation of ROS-positive invadopodia and their ability to degrade extracellular matrix. This study demonstrates that Tks proteins are functional Nox organizer subunits which localize Nox1 activity to invadopodia. These results elegantly show how the presence of different organizer subunits might be responsible for Nox1 localization to distinct subcellular compartments, facilitating spatially-confined ROS production near redox-sensitive targets to initiate specific signaling events. They also suggest that members of the p47phox organizer superfamily may be more generally important in regulating other cellular processes requiring the localized ROS formation.

1648/B27
The Inhibitory Effects of Gan-Lu-Yin on Tumor Cells Induced Angiogenic Activities.
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According to pharmacological functions of the ingredients, Gan-Lu-Yin (GLY), a traditional Chinese herbal formula, has potential to be an anti-angiogenic agent. The purpose of this study was to explore the possible inhibitory effects of GLY on tumor cell-induced angiogenesis. Alcoholic concentrate of GLY was tested on chicken chorioallantoic membrane (CAM) assay and the human umbilical vein endothelial cells (HUVEC) to evaluate the effects of GLY on the cell proliferation, migration and tube formation of vascular endothelial cells with stimulation from serum or tumor cells. Our results showed that 1.5 mg/ml of GLY extract was sufficient to inhibit the HUVEC proliferation, and the treatment of 1.0 mg/ml of GLY extract could markedly reduce cell migration and In Vitro tube formation of HUVEC. In chicken CAM assay, we found that GLY extract could largely reduce the capillary mesh on the CAM of fertilized eggs. Similarly, the inhibitory effects of GLY extract (1 mg/ml) could be also observed on tumor cell-induced HUVEC proliferation and tube formation. Our results indicated that GLY extract may possess anti-angiogenic effects to prevent tumor growth.

1649/B28
Kinetics of p120 Catenin in Human Gingival Cancer Cell Line Treated with ZD1839.
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In the cadherin/catenin cell-cell adhesion complex, p120 catenin (p120) is a very critical element. We previously reported that phosphorylated beta catenin and phosphorylated p120 disrupt the regulation of E- and P-cadherin stability and adherens junction formation in cancer tissues. In order to study the relationship between p120 phosphorylation and the malignancy in a human gingival squamous cancer cell line (BICR78), we investigated the expression of p120 in BICR78 treated with ZD1839 (Gefitinib), which inhibits EGFR phosphorylation. BICR78 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing with 4 ug/ml hydrocortisone and 10% fetal bovine serum. After nearly confluent cells were cultured in serum-free DMEM for overnight, they were incubated with or without 10 ng/ml EGF and/or 1 uM ZD1839 for one hour. After cultivation, we performed Western blotting and immunocytochemical analyses to investigate the
expression of EGFR, Akt, p120 and c-src product, as well as their phosphorylated forms. We observed a decrease in the number of BrdU-labeled BICR78 cells after treatment with ZD1839. Although the expression of EGFR, Akt and p120 showed no changes, the phosphorylation of EGFR, Akt and p120 decreased in BICR78 treated with ZD1839. Expression of c-src product and phosphorylated c-src product also decreased after treatment with ZD1839. EGFR, p120 and phosphorylated p120 were observed to be distributed on the cellular membrane, while phosphorylated EGFR, both forms of c-src product and both forms of Akt were localized diffusely in cellular plasma. These results indicate that the inhibition of phosphorylation in EGFR and the down-regulation of c-src product by inhibition of the Akt-PKB signaling pathway inhibits p120 phosphorylation, thereby controlling the growth of BICR78 cells.

1650/B29
Characterizing Transitional Epithelia Associated with Tumor Formation.
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Epithelial transitional zones are located at the junction of two organs where abrupt transition occurs from one type of epithelium to another. These transition zones can be found in the eye, esophagus, stomach, cervix and anus. In human, these junctions are highly susceptible to tumor formation and our goal is to identify the underlying molecular and cellular basis for this tumor susceptibility. We have developed a novel mouse model of human squamous cell carcinoma in the anorectal transition zone that allows us to investigate the intrinsic properties of these transitional epithelial cells and their interactions with the neighboring cells. We hypothesize that cells within this region are intrinsically more prone to transformation or alternatively, that the environment is permissive for transformed cells. Cancers have recently been shown to contain a population of cell with the properties of stem cells. This observation has led to the “cancer stem cell” hypothesis in which malignant tumors are initiated and maintained by a stem cell population. We used the property that stem cells divide infrequently and consequently can be marked by a repeated administration of Brdu, followed by a chase period, to develop a novel strategy to specifically label live slow-cycling stem cells in epithelial tissues. Using this approach to label live slow-cycling cells, we show that a residence of stem cells exists in the anal transitional zone and that a combination of stem cell markers defines a novel niche for stem cells at the anorectal junction. We hypothesize that transitional stem cells are uniquely susceptible to transformation either due to intrinsic or environmental “niche” differences. Our work provides the first evidence for the existence of stem cells at transitional epithelia and has global relevance in identifying the molecular basis for the dramatically elevated occurrence of aggressive tumors in transition zones. (This work was supported by CCHMC funding)

1651/B30
Specific Cross-talk Between Epidermal Growth Factor Receptor and Integrin αvβ5 Promotes Carcinoma Cell Invasion and Metastasis.
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Tyrosine kinase receptors and integrins play essential roles in tumor cell invasion and metastasis. Here, we show that EGF stimulates metastasis of carcinoma cells via a Src-dependent phosphorylation of p130 CAS leading to activation of Rap1, a small GTPase involved in integrin activation. Specifically, EGF receptor (EGFR)-induced Src activity leads to phosphorylation of critical tyrosine residues within the CAS substrate domain that represent putative binding sites for other adapter proteins known to promote migration, such as Crk and Nck. Phosphorylation of these tyrosine residues is essential for Rap1 and αvβ5 activation. This pathway induces αvβ5-mediated invasion and metastasis in Vivo yet does not influence primary tumor growth or activation of other integrins on these cells. These findings show how cross-talk between a
tyrosine kinase receptor and an integrin involved in carcinoma cell invasion/metastasis and may explain in part how inhibitors of EGFR influence malignant disease.

1652/B31
The Regulation of CD44 Expression in GBM Cells.
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Glioblastoma multiforme (GBM) is a highly aggressive brain tumor as it demonstrates a high growth rate as well as the capacity to invade surrounding brain tissue. These characteristics result in a dismal prognosis for patients with GBM. The current treatment options for GBM tumors involve surgical resection followed by radiation and/or chemotherapy. Disappointingly, GBM tumors are or become resistant to radiation and chemotherapy, and the invasiveness of GBM precludes curative removal of the tumor. GBM cells infiltration has been associated with the transmembrane glycoprotein, CD44, which is the principle cell surface receptor for hyaluronic acid. The molecular mechanisms that regulate CD44 in GBM are not fully explored. However, CD44 protein expression has been associated with an AP-1 transcription factor, fos related antigen 1 (Fra-1), in another aggressive cancer, mesothelioma. Therefore, we investigated the regulation of CD44 expression in human glioma cells by Fra-1. Human GBM cell lines, U-251 and U-1242, were exposed to either epidermal growth factor (EGF, 100 ng/ml) or hepatocyte growth factor (HGF, 50 ng/ml) at varying time points and Western Blot analysis was used to determine the protein expression of CD44 and Fra-1. CD44 and Fra-1 expression levels increased with longer exposures (4 to 24 hr) to EGF and HGF. To determine whether Fra-1 takes part in the regulation of CD44 expression, we utilized transient gene knockdown using siRNA directed against fra-1. fra-1-siRNA1 decreased Fra-1 expression in EGF- and HGF-treated GBM cells. Following HGF stimulation and fra-1-siRNA treatments, CD44 expression also decreased. Surprisingly, EGF stimulation of fra-1-siRNA treated U-1242 GBM cells showed an increase in CD44 expression. These data suggest that Fra-1 regulation of CD44 expression is growth factor-specific in GBM cells.

1653/B32
TGF-beta1 Stiffens Lung Carcinoma Cells in Culture.
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Each tissue is characterized by a unique stiffness which is intimately associated with its specific function. While tissue stiffness is maintained in normal physiological conditions, it becomes abnormally high in solid tumors. Studies in culture have revealed that epithelial cells grown on substrata that are stiffer than their tissue of origin exhibit a loss of differentiation markers, a raise in proliferation and, in some occasions, the acquisition of a migratory phenotype suggestive of epithelial-to-mesenchymal transformation (EMT). The lung is a moderately soft organ with unique elastic properties that are necessary for breathing. However, how the mechanical properties of the lung become altered during tumorigenesis at the cellular level remains largely unexplored. We used Atomic Force Microscopy to measure the Young's modulus (E) - a common indicator of cell stiffness - of cultured human lung carcinoma cell lines. We examined cell lines known to either undergo EMT (A549) or to not undergo EMT (H441) in response to transforming growth factor beta1 (TGF-b1). Cells were cultured for 3 days in serum-free media without or with TGF-b1. Untreated A549 and H441 cells exhibited low E (450 +/- 30 and 1600 +/- 500 Pa, respectively). In contrast, TGF-b1 treatment induced an eight- and three-fold increase in E in A549 and H441, respectively. Because fibrillar collagens are important regulators of tissue stiffness in the lung and in other organs, we examined the expression of collagen I and III by quantitative RT-PCR. TGF-

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b1 treatment induced a fold-increase of collagen I expression comparable to that of E data in both cell lines. In contrast, TGF-b1 had a weak induction of collagen III. Our findings strongly support that TGF-b1 may compromise the normal mechanical properties of the lung microenvironment by stiffening carcinoma cells and promoting expression of collagen I. Collectively these alterations may contribute to lung tumorigenesis by altering the normal balance of forces in the lung microenvironment and ultimately compromising lung function.

Neuronal Diseases II (1654 – 1678)

1654/B33
Modulation of Tau Phosphorylation by Kinase-kinase Interactions.
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Hyperphosphorylated tau protein in the neurofibrillary tangles is one of the biochemical hallmarks in the brains of Alzheimer’s disease patient. Although phosphorylation alters biophysical and biochemical characteristics of a protein, it is hard to assess the significance of phospho-isofoms of tau, since tau contains as many as 79 serine/threonine residues. Interestingly, only a few sites were found to be phosphorylated in normal adult tau. Tau, a microtubule (MT)-associated protein, regulates MT remodeling as a protein complex together with p35-cyclin-dependent protein kinase (p35-CDK5) and glycogen synthase kinase 3beta (GSK3beta). We focused on regulation of tau by combined action of tau kinases. We conducted an in-vitro kinase assay using recombinant human tau p35-CDK5, GSK3beta, and cAMP dependent protein kinase a (PKA). Tau phosphorylation was analyzed by Western blot. We found that a specific sequence and combination of kinases evoked not only a stimulatory but also an inhibitory effect on tau phosphorylation. Striking enhancement of tau phosphorylation by GSK3beta was induced by preincubation of either p35-CDK5 or that of PKA. Tau phosphorylation was also stimulated by simultaneous application of PKA and GSK3beta. on the other hand, tau phosphorylation with p35-CDK5 was diminished by preincubation of tau with GSK3beta. This inhibitory effect suggests that phosphorylation of tau protein by p35-CDK5 can be maintained at physiological level by GSK3beta. In this case, close association of tau, p35-CDK5, and GSK3beta with MT undoubtedly contributes to fine-tuning of physiological level of phospho-isofoms of tau. Preincubation of tau with PKA inhibited further phosphorylation of tau by p35-CDK5. These evidences suggest that the three kinases work together to maintain physiological level of tau phosphorylation in a sequence-dependent manner. These negative regulations by the kinases on tau phosphorylation may play an important role on protection against abnormal hyperphosphorylation of tau.

1655/B34
Ubiquitination of the Polyglutamine Disease Protein Ataxin-3 Enhances its Deubiquitinating Activity and Regulates its Cellular Functions.
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Posttranslational modification of proteins by ubiquitin regulates many cellular pathways. Deubiquitinating enzymes reverse protein ubiquitination and are important regulators of protein fate, interactions and localization. The deubiquitinating enzyme ataxin-3 functions in protein quality control and has a polyglutamine tract that, when expanded, causes the neurodegenerative disease Spinocerebellar Ataxia Type 3 (SCA3). Ataxin-3 is ubiquitinated in cells, and here we show that this modification directly enhances ataxin-3’s ability to cleave ubiquitin chains in vitro. Levels of endogenous, ubiquitinated ataxin-3 increase when the proteasome is impaired or the unfolded protein response is induced. The catalytic activity of polyglutamine-expanded (pathogenic) ataxin-3 is also enhanced by ubiquitination. Intriguingly, pathogenic ataxin-3 is more heavily ubiquitinated than wild type ataxin-3 in a mouse model of SCA3. Lysine (K) at position 117, which resides near a recently identified ubiquitin-binding site, is the most commonly
ubiquitinated residue on ataxin-3. Ubiquitination at K117 is sufficient to enhance activity of ataxin-3; other lysines are not ubiquitinated efficiently and, when ubiquitinated, do not lead to enhanced activity. Ubiquitination of K117 is also important for ataxin-3's ability to restrict the length of ubiquitin chains on proteins in vitro, and to regulate levels of a proteasomal substrate, CFTRΔ508, in cells. Collectively, our data indicate that the activity of this disease-related deubiquitinating enzyme is regulated by ubiquitination during the cellular response to certain types of stress and provide insight into the mechanism underlying ataxin-3's activation by ubiquitination.

1656/B35
Dynamin-2 Cellular Function and the Consequences of Mutations Linked to Centronuclear Myopathy and Charcot-Marie-Tooth Disease.
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Dynamin (Dyn), a multidomain GTPase, is best studied for its role in clathrin-mediated endocytosis (CME); however, recent studies have suggested that it also functions in other endocytic and intracellular trafficking events. The human genome encodes three isoforms of dynamin with different tissue specific expression; among them, Dyn2 is the only ubiquitously expressed isoform, and Dyn1 is the major dynamin in neuronal cells. Mutations in Dyn2 have been linked to autosomal dominant centronuclear myopathy (CNM) and autosomal dominant Charcot-Marie-Tooth disease type 2B (CMT2B). Neither the nature of the biochemical defect nor the cellular processes affected in mutant cells that lead diseases are known. The CNM-associated mutations cluster in the middle domain of Dyn2, while most CMT-associated mutations map to the PH domain. More recent studies have suggested that the phenotypes of Dyn2 related CNM and CMT overlap, and Dyn2 mutants causing CMT have been mapped to the middle and Pro/Arg-rich domains. We expressed Dyn2 mutants associated with CMT and CNM in previously characterized conditional Dyn2 knock-out (KO) cells (Liu et al., 2008, MBC 19:5347-5359) in the presence and absence of endogenous Dyn2 to analyze their localization, ability to compensate the various KO phenotypes and function as dominant-negative alleles. We find that CNM mutations affect Dyn2 localization, but surprisingly are fully active in restoring CME. CMT mutations we have studied do not affect localization and none have dominant-negative effects on CME. Together these data suggest that the disease-causing mechanisms of Dyn2 for CNM and CMT2B are unlikely to be due to defects in CME. We are currently examining other isoform specific functions to identify the disease-causing mechanism.

1657/B36
Acute Changes in Protein Expression and Phosphorylation Following Loss of Merlin Function in an In Vitro model of Neurofibromatosis Type 2.
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Neurofibromatosis Type II (NF2) is an inherited neurological tumor disorder caused by mutations in the nf2 gene, which codes for the tumor suppressor protein merlin. Loss of merlin function promotes formation of bilateral vestibular schwannomas (VS) on cranial nerve VIII that causes hearing loss. Merlin is a tumor suppressor that regulates cell-matrix adhesion, cell-cell contact and proliferation through its interactions with integrins, cadherins and receptor tyrosine kinases (RTKs), specifically members of the EGF and ErbB family. Activation of β1-integrin and erbB2 receptors promotes p21-activated kinase and protein kinase a dependent phosphorylation of merlin at serine 518. This modification inhibits its tumor suppressor activity. Our laboratory obtained transgenic mice with loxP sites flanking exon 2 of the nf2 gene (nf2floxflox2). Exon 2 encodes a paxillin-binding domain (PBD1) necessary for localization of merlin to the plasma membrane. SCs were isolated from these mice and exon 2 was excised In Vitro using an adeno virus encoding Cre recombinase (Cre). Because merlin lacking PBD1 has a reduced half-life, mouse nf2floxflox2 SCs infected with adeno-Cre virus should have low levels of non-functional merlin. We aimed to establish merlin expression and phosphorylation level in mouse nf2floxflox2
SCs 3, 4 and 5 days following infection with adeno-Cre virus. Our results show that within 5 days of deletion of exon 2, merlin expression is reduced by nearly 50 percent. Interestingly, merlin phosphorylation increases 2-fold indicating the residual merlin is no longer active as a tumor suppressor. We conducted a series of western blots comparing mouse nf2lox2/fox2 and wild type SCs to analyze immediate changes in phosphorylation of RTKs and intracellular signaling proteins subsequent to the loss of merlin function. We show that by 5 days there is a 30 percent increase in phosphorylation of AKT. This increase is in line with reported increase in AKT activity in human VS. These changes in protein expression and phosphorylation support the usefulness of this cell system as an In Vitro model of NF2.

**1658/B37**

**Molecular Basis of Ethanol on Eye and Neural Crest Development in Zebrafish.**

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Our laboratory has begun to use zebrafish to examine the molecular basis of fetal alcohol syndrome (FAS), with an emphasis on eye development and cranial neural crest cell migration and differentiation. Two developmental hallmarks of fetal alcohol exposure are microphthalmia (small eyes) and craniofacial malformations associated with perturbed neural crest cell development. Recent studies from our laboratory have shown that morpholino knockdown of agrin in zebrafish results in microphthalmia, which is mediated by disrupted mediation of Fgf function by agrin (Liu et al, 2008, Dev. Neurobiol. 68, 877-898). Since Fgf function has been reported to be a target of fetal ethanol exposure we initiated studies to assess the role of agrin in FAS-associated microphthalmia. Our studies show that agrin mRNA expression is reduced in zebrafish embryos exposed to ethanol, and subthreshold doses of agrin morpholino and ethanol act in concert to further reduce agrin gene expression and produce microphthalmia during zebrafish retinal development. Using the neural crest cell marker crestin to assess cranial neural crest cell migration in response to embryonic ethanol exposure, we demonstrate that neural crest cell migration into head and eye of zebrafish embryos is markedly reduced with ethanol treatment, while crestin gene expression appears normal in trunk regions. Whereas agrin knockdown potentiates ethanol effects in eye development, cranial neural crest cell migration is unaffected by agrin knockdown. We are currently extending these studies to examine the role of retinoic acid in agrin- and ethanol-dependent microphthalmia, as well as cranial neural crest cell development, as retinoic acid is a recognized target of fetal alcohol exposure. These studies are intended to use the zebrafish model system to further our understanding of the molecular basis of FAS, with our results suggesting that agrin plays a role in ethanol-mediated disruption of eye development. Supported by NIH grants MD000175 and NS33981.

**1659/B38**

**Stereological Analysis of the Brain of Cystatin B -Deficient Mouse Model for Progressive Myoclonus Epilepsy, EPM1.**

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Progressive myoclonus epilepsy of Unverricht-Lunborg type (EPM1) is an autosomal recessive disorder caused by loss-of-function mutations in the Cystatin B (CSTB) gene. It is characterized by age of onset at 6-16 years, stimulus-sensitive myoclonus, tonic-clonic seizures and ataxia. Cstb-deficient mice develop a phenotype resembling that of human EPM1 patients: myoclonic seizures develop by 1 month of age and progressive ataxia by six months of age. Neuropathological findings include atrophy and gliosis especially in cerebellum with apoptotic death of cerebellar granule cells. The aim of this study is to determine the precise sequence of the neurodegenerative events in Cstb-deficient brain, particularly in the earliest stages in which the central nervous system is impacted by the disorder. We have analyzed neuronal and glial
components of Cstb-deficient mouse brain at different stages of disease progression using unbiased stereological methodology. Cstb-deficient brains show widespread and progressive regional atrophy of the cerebral cortex and cerebellum. Cortical thinning is similar in all cortical areas, as is the atrophy of individual cerebellar layers. Widespread astrogliosis and microgliosis can be seen already in 1-month-old Cstb-deficient mice, with a distinct distribution that is preserved throughout the disease progression. In cortex, gliosis is concentrated in outer and inner layers while middle layers are more preserved. This pattern is seen in all cortical areas, with the exception of the cingulate cortex that shows massive gliosis in older mice. Other affected areas include midline and posterior thalamic nuclei, amygdaloid nuclei, claustrum and substantia nigra. In contrast, the hippocampus is almost totally spared. Interestingly, activated microglia in young mice show several different phenotypes, from ramified to amoeboid, in the same affected areas, but this variation disappears with disease progression. Our findings provide novel information about the neuropathological changes due to CSTB deficiency at regional and cellular level. Importantly, our data suggest a microglial involvement in the neuronal dysfunction and selective neuronal death in EPM1.

1660/B39
Normal Cerebral Cortical Development Requires Dystroglycan in the Brain but not the Meninges.
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The root cause of Type II lissencephaly found in cerebral cortical malformations of congenital muscular dystrophies (CMDs) is caused by disruptions of the pial basement membrane. The pial basement membrane (PBM) is sandwiched between the glia limitans of the brain and the meninges. Its integrity is dependent on the functions of two cellular receptor signaling systems with the extracellular matrix, integrin and dystroglycan. Earlier work by Beggs et al., (Neuron, 40:501-514, 2003) show that focal adhesion kinase is required both in the brain and in the meninges for the maintenance of the PBM integrity, suggesting that integrin signaling is required in both the brain and the meninges. With regard to dystroglycan signaling, it is not clear what cell types are required for PBM maintenance. We hypothesize that dystroglycan is required in the glial endfeet, not meninges cells. To test this hypothesis, we generated neural- and meninges-specific knockout of dystroglycan with Nestin-cre (Graus-Porta et al., Neuron, 31:367-379, 2001) and Wnt1-cre (Chai et al., Eur.J.Biochem, 127:1671-1679, 2000) transgenic mice. Neural-specific tissue deletions produced several severe phenotypes including: breaches in PBM, overmigration of neurons, and cortical lamination defects. By contrast, meninges-specific deletions did not produce an observable abnormal phenotype. These findings indicate that unlike integrin signaling, dystroglycan signaling is required in the developing neural tissue, but not the meninges for maintaining PBM integrity and normal cortical development. Our work implies that future gene therapy of these related disorders should be directed in the neural tissue to prevent/alleviate type II lissencephaly.

1661/B40
Hsp70 Overexpression Inhibits ER Stress-induced Apoptosis by Enhancing XBP1 Signalling.
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Endoplasmic reticulum (ER) stress-induced apoptosis, caused due to the accumulation of unfolded proteins in the ER, is been associated with various diseases collectively called conformational diseases. The major heat shock protein, Hsp70 is well known to be an effective inhibitor of apoptosis. Hsp70 interacts with and affects several different molecules in the apoptotic pathway. Hsp70 has been shown to interfere with recruitment of procaspase-9 into the apoptosome, inhibit the release of the proapoptotic protein Smac/DIABLO from mitochondria and to sequester AIF. In this study we analysed the role of Hsp70 in preventing ER stress-induced
apoptosis. We have used PC12 cells overexpressing Hsp70 to study the mechanisms by which Hsp70 prevents ER stress-induced apoptosis. Our results demonstrate that Hsp70 inhibits ER stress-induced apoptosis upstream of mitochondria. This was shown by reduced cytochrome c release and loss of mitochondrial membrane potential in Hsp70 overexpressing cells. Furthermore, Hsp70 overexpression potentiated the production of spliced X Box Binding Protein-1 (XBP-1) which is downstream of Inositol Requiring Enzyme-1 (IRE1) signaling and leads to increased transcriptional upregulation of several UPR target genes downstream of spliced XBP-1 like EDEM1, ERdj4 and P58IPK. Overexpression of dominant negative IRE1 specifically compromised the inhibition of ER stress-induced apoptosis by Hsp70. Taken together our results show that Hsp70 prolongs the production of spliced XBP-1 and protects the cells from ER stress-induced apoptosis by regulating IRE1-XBP1 pathway of unfolded protein response.

1662/B41
Determinants of SMN Cleavage by Calpain and Regulation of SMN Function.
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Spinal muscular atrophy (SMA) is a leading genetic cause of infant mortality. SMA is caused by mutations in the survival motor neuron 1 (SMN1) gene, which functions in the biogenesis of small nuclear ribonucleoproteins (snRNPs). It is not yet clear how reduced levels of SMN protein result in neuromuscular disease. We have recently demonstrated that the SMN complex localizes to the Z-discs of both skeletal and cardiac muscle sarcomeres, indicating additional tissue-specific functions of SMN. Further, loss of Smn function affected the organization of Z-discs in SMA mice. Consistent with the localization of Smn to Z-discs and the involvement of calpains in muscle maintenance, we have demonstrated that SMN is a proteolytic target of calpains. Calpains are calcium activated cysteine proteases that are expressed ubiquitously. They typically perform limited cleavage of their substrates, regulating substrate activity. Here, we show that calpain cleavage of SMN produces ~28 kDa and ~10 kDa fragments, which can be detected both In Vitro and in vivo. In addition, we demonstrate that SMN cleavage is restricted to the cytoplasm, suggesting that calpain cleavage does not serve as a release mechanism from Cajal bodies. Sequence analysis of SMN reveals that the protein contains a strong PEST motif, a determinant of protein stability often found in calpain substrates. Importantly, deletion of this motif or the predicted target site (which often lies outside the PEST motif) renders the protein refractory to calpain cleavage. Mutation of a known caspase cleavage site within SMN (D252A) does not affect cleavage by calpain. Mapping of the precise calpain cleavage site and characterization of the determinants of SMN cleavage are underway. Additional studies aimed at understanding the biological and/or etiological significance of the cytoplasmic calpain cleavage of SMN will be discussed.

1663/B42
OPA1 Plays a Critical Function in Ca2+ Homeostasis.
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Optic Atrophy 1 (OPA1) mutations cause dominant optic atrophy (DOA) with retinal ganglion cell (RGC) and optic nerve degeneration. Why RGCs selectively degenerate in DOA is unknown. Moreover, the mechanism underlying RGC cell death remains elusive. To embark on these questions we reduced OPA1 protein in cell lines and RGCs by RNA interference (RNAi). OPA1 loss triggered mitochondrial fragmentation, oxidative phosphorylation inhibition, ATP decrease, and sensitization to apoptotic insults. While the mitochondrial cristae junctions remain of normal size, we observed profound cristae depletion in cells lacking OPA1 using EM tomography. Concomitant with this ultrastructural change, mtDNA copy numbers were reduced. Importantly, mitochondria of OPA1 knockdown cells buffer Ca2+ less effectively and are sensitized to
mitochondrial permeability transition (MPT). In addition, OPA1 siRNA cells exhibit impaired Ca2+-mediated NADH oxidation. Last, while OPA1 loss in RGCs had no impact on mitochondrial shape, exposure of RGC with reduced OPA1 are sensitized to neurotransmitter glutamate induced delayed Ca2+ deregulation (DCD) and neuronal cell death by excitotoxicity. Thus, while OPA1 mediates mitochondrial fusion, maintenance of mtDNA, oxidative phosphorylation, and cristae formation; it has additional functions in Ca2+ homeostasis. Supported by: R01 NS047456, R01 EY016164, R01 NS055193 (to EBW), P41RR04050, R01 NS14718 (to MHE).

1664/B43
New Insights into Cellular Maturation and Transport of the Neuronal Disorder Protein CLN5.
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Neuronal ceroid lipofuscinoses (NCLs) collectively constitute the most common group of inherited neurodegenerative disorders in children. The NCL diseases share clinically and pathologically many similar features, although NCLs are genetically very heterogeneous. To date, mutations in eight different genes have been reported to result in NCL. The Finnish variant late infantile form of NCL (vLINCLFin) is caused by mutations in the CLN5 gene. To date, 18 disease-causing mutations in this gene have been reported (NCL mutation database, http://www.ucl.ac.uk/ncl). The gene encodes a lysosomal glycoprotein with unidentified function. In this study we have characterized the CLN5 protein in more detail, focussing on the processing, maturation and its trafficking. We demonstrate that the maturation of CLN5 involves a cleavage step in the endoplasmic reticulum (ER). Transient transfections of GFP-CLN5 constructs showed that the N-terminus remains in the ER and the mature polypeptide is transported to lysosomes. A novel C-terminal CLN5 antibody detected 50 kDa and 60 kDa polypeptides in Western Blot analysis of the stably transfected CLN5 cell line. Thorough analyses indicated that the 50 kDa polypeptide corresponds to the heavily glycosylated, mature CLN5 protein. We also provide the first evidence that CLN5 can traffic to lysosomes without using the mannose-6-phosphate receptor (MPR) pathway, since the over expressed mCln5 was found in the Lamp-1 positive lysosomes in MPR-deficient mouse fibroblasts. Transient expression of CLN5 constructs carrying vLINCL disease-causing mutations in HeLa and SH-SY5Y cells demonstrated that all all the mutations disturb the lysosomal trafficking of CLN5. The level of lysosomal targeting does not, however correlate to disease onset, indicating that CLN5 may also function outside lysosomes. These data increase the understanding on the basic properties of the CLN5 protein, required for the characterization of the consequences of disease mutations and for the development of future therapies.

1665/B44
Characterization of Optineurin.
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Objective: to examine the basic characteristics of optineurin, a gene linked to glaucoma, a major blinding disease. Methods: Total lysates from rat retinal ganglion RGC5 cells were subjected to N- or O-deglycosylation treatment or membrane protein extraction followed by Western blotting. The phosphorylation status was evaluated after immunoprecipitation. for localization of the endogenous optineurin, RGC5 and human retinal pigment epithelial (RPE) cells were double stained with anti-optineurin and anti-GM130 antibodies. To determine the turnover rate, lysates from cells treated with cycloheximide were harvested at various time points for immunoblotting. In addition, RGC5 and RPE cells transfected to express optineurin-GFP were treated with nocodazole and were then washed for nocodazole removal and recovery. Native blue gel
electrophoresis and Western blotting were performed to determine whether optineurin interacts with itself and whether complexes were formed between optineurin and its interacting molecules. Results: While phosphorylated, optineurin was neither N- nor O-glycosylated, and was by itself not a membrane protein. In RGC5 and RPE cells, optineurin exhibited a diffuse, cytoplasmic distribution but a population of the protein was associated with the Golgi apparatus. Turnover experiments showed that the endogenous optineurin was a relatively short-lived protein with a half life of approximately 8 hr. Native blue gel electrophoresis revealed that the endogenous optineurin formed homohexamers. Optineurin also interacted with Rab8, myosin VI, and transferrin receptor to assemble into super molecular complexes. When overexpressed, optineurin-GFP fusion protein formed punctuate structures termed foci in the perinuclear region and induced Golgi fragmentation. The foci formation was dependent on the integrity of microtubules. Conclusion: The present study illustrated the basic characteristics including the glycosylation and phosphorylation status of optineurin. The demonstrations that optineurin was an aggregation-prone protein and that the foci formation was microtubule dependent bear similarities to features documented in neurodegenerative diseases, supporting a neurodegenerative paradigm for glaucoma.

1666/B45
iCRE-CVB3: A Molecular Reporter Virus to Identify Sites of Persistent Coxsackievirus Infection.
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The central nervous system (CNS) of neonatal mice is highly susceptible to infection by group B coxsackieviruses, and neural stem cells are preferentially targeted following infection. Infectious coxsackievirus B3 (CVB3) can be isolated from the CNS of neonatal mice up to ten days post-infection (PI). Additionally, the genome of the virus can persist within the neonatal CNS up to ninety days PI as determined by real time RT-PCR, despite the lack of infectious virus. The aim of the current study was to generate a recombinant CVB3 expressing an improved eukaryotic codon version of CRE recombinase (iCRE-CVB3) which can catalyze the permanent expression of a reporter gene within infected cells. iCRE-CVB3 will be used in combination with the Z/EG dual reporter transgenic mouse line which constitutively expresses the lacZ gene in absence of CRE recombinase. However in the presence of CRE recombinase, a functional DNA rearrangement activates the expression of GFP. Thus, GFP is permanently expressed specifically within infected cells for the lifetime of the host organism. Recently, we have generated a high tittered stock of iCRE-CVB3 following transfection of In Vitro transcribed viral RNA generated from our iCRE-CVB3 infectious plasmid clone. We have recently isolated neurospheres from Z/EG transgenic mice. These primary neural stem cells can be continuously passaged, express the lacZ gene, and can be induced to express eGFP following transfection of a plasmid expressing CRE recombinase. Experiments underway will determine if infection of Z/EG neurospheres with our iCRE-CVB3 viral stock will lead to iCRE-mediated GFP expression. Eventually, Z/EG transgenic mice will be directly infected with iCRE-CVB3, and sites of viral persistence will be identified by the continued expression of GFP. Our ultimate goal is to use our iCRE-CVB3 in combination with these CRE-reporter mice to study the link between microbes and chronic diseases such as diabetes, myocarditis, and neurodegeneration. Utilizing our novel system, we expect that the connection between prior infection and eventual disease may become firmly established.

1667/B46
Development of a Technique that Uses Total Genomic DNA and PCR to Genotype Knockout Strains of Caenorhabditis elegans.
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Knockout lines of C. elegans are made by mutagenizing the worms. To obtain a totally pure line with only one gene knocked out, the original worms must first be backcrossed 3-4 times to the N2
wildtype. Some investigators then genotype the worms by performing single worm PCR but this procedure can give unreliable results. Here we show an alternative technique where we use a half of a brood of worms and use this as a source to phenol-chloroform extract genotypic DNA. This method can be helpful where the PCR is problematic and the use of only one worm would not result in sufficient DNA to perform PCR. Briefly, we first bred 3 wild type males with 1 knockout L4 hermaphrodite. If males are present in large numbers, indicating that breeding occurred, we transferred L4 hermaphrodites to individual plates and let them lay eggs for 1-2 days before transferring the parent to a new plate. We then performed PCR on the first plates to confirm that heterozygotes were present. Of the offspring produced on the second plate, 25% should be knockouts. To confirm this, L4 hermaphrodites were transferred to their own plate, allowed to lay eggs and the parent was then transferred again to a new plate. Following PCR, the wild type worms produced an 1100 bp fragment, the knockouts produced a 500 bp fragment and the heterozygotes produced both an 1100 bp and a 500 bp fragment. The homozygous knockout worms were then bred back with the wild type and this procedure was repeated 3 more times. We now have a pure population of the T27A3.1 knockout line which is the homologue for the Huntington Associated Protein 1 (HAP1) gene that is found in humans. These worms are now being used in behavioral and biochemical studies.

1668/B47
PolyQ-htt Alters TrkB Retrograde Dendritic Transport in the Striatum.
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Huntington's disease (HD), a neurodegenerative disorder leading to death in 10 to 20 years, is due to an expansion of the polyglutamine (polyQ) stretch in the huntingtin protein (htt). HD is characterized by a specific neuronal cell death in the striatum and the cortex. The mechanisms by which huntingtin induces dysfunction and death of neurons in the brain are not clearly understood but these mechanisms could involve both the gain and the loss of huntingtin function. Using videomicroscopy, we unravelling a function of huntingtin in the microtubule-based transport of neurotrophic factors such as Brain Derived Neurotrophic Factor (BDNF). BDNF is a neurotrophic factor synthesized in the cortex and transported to the striatum through cortico-striatal afferences. Previously, we showed that polyQ-htt reduces velocity and BDNF neurotrophic support to the striatum leading to cell death. However, nothing is known about the consequences of the mutation on transport of BDNF and its receptor, TrkB, in the striatum, the target neurons. We demonstrate here that htt is involved in TrkB transport as down regulation of htt expression by siRNA results in a significant reduction of TrkB vesicular transport. Next, we investigated the role of the polyQ-htt by cotransflecting striatal cell lines expressing the wild-type or polyQ-htt with TrkB-RFP. We showed that TrkB vesicles velocity is reduced in polyQ-htt cells. However, these cell lines are not polarized as neurons. We therefore used rat primary striatal neurons cotransfected with a N-terminal form of htt (480 amino acids) expressing a normal or polyQ stretch and TrkB-RFP and analyzed selectively transport in dendrites using MAP2-GFP that localizes specifically in dendrites. We observed a reduced velocity of the TrkB vesicles in dendrites of polyQ-expressing neurons. Finally, we also observed a reduction of TrkB dendritic velocity in mouse primary striatal neurons HD knock-in mice that express polyQ-htt at the endogenous level. In conclusion, our data demonstrate that TrkB transport is altered in the dendrites of striatal neurons in HD.

1669/B48
Morphological and Molecular Dissection of the γ-Secretase-independent Function of Presenilins in Endosomal Trafficking.
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Presenilins (PSEN1&2) form the catalytic entity of the γ-secretase complex, involved in the cleavage of type I transmembrane proteins, including APP and Notch. Besides this catalytic role, PSENs harbor other functions related to protein transport and turnover, β-catenin signaling, and calcium homeostasis. We previously identified a role for PSEN1 in the fusion of ICAM-5 positive degradative organelles with lysosomes (Esselens, JCB, 2004). The additional more recent observation that PSEN KO mouse embryonic fibroblasts (MEFs) present distinct morphological features, including extensive lamillipodia formation, fostered our hypothesis that this might be caused by an impairment in distinct endosomal sorting route(s) due to PSEN deficiency. As a consequence, PSEN1KO and PSENdKO MEFs migrate faster in wound healing assays and adhere less well as compared to wild type MEFs. These features could not be mimicked by γ-secretase inhibitors, but can be rescued by dominant negative aspartate mutants, corroborating the relation to the γ-secretase-independent functions of mainly PSEN1. Interestingly the morphology of PSENdKO MEFs is reminiscent of caveolin1 deficiency and along this line we observed a depletion of caveolin 1 at the cell surface. Instead, most endogenous caveolin 1 accumulates intracellularly, indicating that cell migration and adhesion problems could be caused by a failure of caveolin 1 to redistribute from intracellular caveosomes. Indeed, using a suspension-adhesion assay, we can show that the redistribution, but not the internalization, of cholera toxin B, a marker for lipid rafts is significantly affected in PSENdKO MEFs. In support of selective endosomal routes affected in PSENdKO MEFs, we noticed no difference in transferrin receptor internalization; on the contrary we found accumulations of EGFR in acidic compartments. We are currently identifying the specific internalization/redistribution routes that are corrupted in these MEFs. Since endosomal dysfunction is an early feature in the etiology of Alzheimer's disease, identification of the affected endocytic routes may lead us to new molecular targets restoring PSEN function.

1670/B49
Alterations in the Properties of Oligodendrocytes in the Neuronal Ceroid Lipofuscinosis CLN8.
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The neuronal ceroid lipofuscinoses (NCLs; CLN1-CLN10), a group of inherited lysosomal storage disorders, are the most common cause for pediatric neurodegeneration. Their clinical course includes progressive mental and psychomotor retardation, epilepsy, blindness and premature death. Mutations in the CLN8 gene account for two types of NCL: Northern epilepsy in Finland and the late infantile variant enriched in the Mediterranean. The motor neuron degeneration mouse, Cln8mnd is used as a model to study CLN8 disease. Mechanisms of neuronal dysfunction in the CLN8-related NCLs are largely unknown. Based on sequence homology, CLN8 is linked to a TLC (TRAM, LAG1, CLN8) superfamily of proteins, which have been proposed to have a role in lipid biosynthesis, metabolism and sensing. We have shown perturbed sphingolipid metabolism in Northern Epilepsy patient brains. In addition, we have shown a decrease in myelin-specific galactolipids in Cln8mnd mouse brains as well as delayed myelination. This was explained, at least partially, by the reduced activity and mRNA level of galactolipid-synthesizing enzyme UDP-galactose:ceramide galactosyltransferase (Ugt8A). Oligodendrocytes are the main Cln8 expressing cells in the CNS and responsible for synthesis of galactolipids. In this study, we have used neural progenitor cells from Cln8mnd to investigate whether the reduced levels of galactolipids have consequences on oligodendrocyte development and maturation. Our results show enhanced neural progenitor proliferation and increased differentiation towards glial fates, which appear to be explained by disturbed growth factor signaling. In vivo, by real-time PCR, we have observed increased oligodendrocyte marker expression in Cln8mnd brains. To determine whether the amount of oligodendrocytes is increased in Cln8mnd mice, we are stereologically quantifying the number of oligodendrocytes at different maturation stages in the brains of Cln8mnd. In conclusion, our study indicates that oligodendrocytes is the key cell type in the CLN8 disease and propose a possible link between CLN8 and Ugt8A. These findings are critical for
understanding the pathomechanisms of CLN8 disease as well as the physiological function of the CLN8 protein.

1671/B50
Overexpression of minibrain Models Down Syndrome in Fruit Flies (Drosophila melanogaster).
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Down Syndrome (DS) occurs in about 1 in every 700 live births, and includes cognitive impairment, learning and memory deficits, lower brain weight, reduced neuronal density, number and volume, and hypotonia. The DS candidate gene, dual-specific tyrosine-regulated kinase 1a (dyrk1a) encodes a protein that can phosphorylate serine and threonine residues in a variety of substrates, and is connected to neural differentiation and neurogenesis. Studies of the Drosophila melanogaster homologue minibrain (mnb) reveal learning and memory deficits in mutant flies. We show that overexpression of mnb, using the UAS-GAL4 system of gene expression, produces DS-like phenotypes. Ubiquitous overexpression of mnb decreases the number of ommatidia in the eye and also results in decreased locomotor ability. When mnb is overexpressed in the eyes, neurons and neuroblasts, the development of adult flies is slightly delayed. Overexpression of mnb models DS in fruit flies and may elucidate cellular pathways important in DS.

1672/B51
Calnexin Myelopathy: A Murine Model of Neuropathy.
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Calnexin, a ubiquitous endoplasmic reticulum (ER) chaperone, is a critical component of quality control in the protein folding pathway. Calnexin, along with calreticulin and protein disulfide isomerase ERP57, is responsible for ensuring that only properly folded and assembled proteins exit the ER. We have generated calnexin gene-deficient mice that demonstrate an unanticipated and highly specific peripheral neuropathy. While popular conception depicts a critical role of calnexin in the synthesis, folding and maturation of immune system components and immune system function, a comprehensive analysis revealed calnexin-deficiency had no discernible effects on most systems, including immune function. Instead, the mice have perturbed motor function characterized by gait disturbance, lower limb dysfunction and ataxia. Morphological analyses revealed calnexin-deficient mice have loose, decompacted myelin sheaths. Nerve conduction velocities are reduced in the absence of calnexin, consistent with dysmyelination and reminiscent of neuropathies like Charcot-Marie-Tooth. Neuronal growth, number and function appeared unimpaired in the calnexin-deficient mice. Biochemical investigations revealed that calnexin-deficient mice have downregulated expression of P0 and PMP22, key myelin proteins required for the formation and maintenance of compact myelin sheaths. Additionally, the absence of calnexin results in misfolded PMP22 and P0 that are nonfunctional in the context of their adhesive properties. This work highlights a specific role for calnexin in neuronal systems and provides a novel candidate for investigation in neuropathies.

1673/B52
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The mutations of long-chain acyl-CoA synthetase 4 (ACSL4) result in non-syndromic X-linked mental retardation (MRX). ACSL4 converts long chain fatty acids to acyl-CoAs, and is indispensable in lipid metabolism and various signaling pathways. We previously showed that ACSL4 is required for Dpp BMP production and visual circuit wiring in the brain (Zhang et al., Human Molecular Genetics 2009). Here we use the Drosophila neuromuscular junction (NMJ) as a model system to further dissect its role in the nervous system and the pathogenesis of ACSL4-associated MRX. Drosophila Acsl mutants display obvious aggregates of axonal cargoes marked with synaptic proteins CSP and NC82, but the axonal distribution of mitochondria is normal. Live imaging of axonal transport of synaptotagmin-GFP labeled cargoes further demonstrated that Acsl mutations impaired retrograde but accelerated anterograde transport of synaptic vesicle precursors, while the transport of mitochondria labeled by Mito-GFP was not affected. We also found that the synaptic terminals on the anterior abdominal muscles are overgrown while those on the posterior abdominal muscles are dystrophic. Membrane trafficking assay showed an increased intensity of newly synthesized membrane protein CD8-GFP in the NMJs on the anterior segments while a decreased level of CD8-GFP in the NMJs on the posterior segments. In addition, the synaptic structural defects are accompanied by decreased neurotransmission. Together, our findings demonstrate that Acsl is required for axonal transport and plays a critical role at NMJ synapses. The axonal and synaptic defects we revealed in Drosophila Acsl mutants may account for the pathogenesis of ACSL4-related MRX.

1674/B53
Retinal Protection from Ischemia-Reperfusion Injury through Pharmacological Induction of Heme Oxygenase-1 by Cobalt Protoporphyrin.
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OBJECTIVE. To investigate the protective effects of cobalt protoporphyrin (CoPP), a potent heme oxygenase-1 (HO-1) inducer, in a rat model of ischemia-reperfusion injury. METHODS. Rats pretreated with intraperitoneal injection of CoPP (5mg/kg body weight) 2 days before ischemia insult were subjected to retinal ischemia induced by increasing intraocular pressure to 130 mmHg for 60 minutes followed by 6 hours, 12 hours, 24 hours, and 7 days of reperfusion. Rats pretreated with Zinc protoporphyrin (ZnPP) (50 μmol/kg body weight) or vehicle ( phosphate-buffered saline ) were served as control groups. The protective effects of CoPP were evaluated by determining the morphology of the retina, and counting the survival of retinal ganglion cells (RGCs) by retrograde fluorogold (FG) labeling at 7 days of reperfusion as well as measuring apoptosis in retinal layers by using TdT-dUTP terminal nick-end labeling (TUNEL) at 24 hours of reperfusion. RESULTS. Pharmacological induction of HO-1 by CoPP led to HO-1 expression in retinal ganglion cells, inner plexiform layer, inner nuclear cells and photoreceptors. HO-1 overexpression alleviated the apoptosis in retina at 24 hours of reperfusion, preserved retinal ganglion cells and attenuated reduction of inner retina thickness 7 days after ischemia-reperfusion injury. Concurrently, overexpression of HO-1 was associated with inhibition of caspase 3, p53, NF-κB, iNOS, and increased expression of bcl-xl. CONCLUSIONS. Overexpression of HO-1 by pharmacological induction protected retina from subsequent cellular damage caused by ischemia-reperfusion injury through anti-apoptotic and anti-inflammatory effects.

1675/B54
Defective Tubulo-Vesicular Transport as a Basis of Niemann Pick Type C Disease.
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Niemann Pick Type C (NPC) disease is a recessive neurodegenerative disorder that induces pathological accumulations of cholesterol and other lipids. Of the two genes implicated in NPC disease (NPC1 and NPC2), mutations in NPC1 account for 95% of cases. NPC1 is known to reside in late endosomes and lysosomes, however the specific function of NPC1 remains elusive. Live-cell imaging of fluorescently-tagged constructs reveals that wild-type NPC1 incorporates into
dynamic tubulo-vesicular membranes (TVMs) that emanate from large spherical vesicles (LSVs). These dynamic TVMs elongate and contact other organelles, facilitating the transfer of membrane contents including cholesterol. In contrast, live-imaging of mutant NPC1 reveals a loss of tubulation in these membranes. We propose that NPC1 TVMs are responsible for the sorting and transfer of cholesterol between membrane compartments and mutant phenotypes reflect a loss of TVM formation. Membrane tubulation requires a combination of wild-type NPC1, an extended and dynamic microtubule (MT) array, and a class of MT-binding proteins known as “tip-trackers”. We hypothesize that that “tip-tracking” proteins might serve to link TVMs to dynamic MTs, thereby providing a mechanism for TVM extension. We tested this hypothesis using drugs that regulate the dynamics of the tip-tracking protein dynactin, assessing tubulation of NPC1 membranes. Both rapamycin and forskolin enhance phosphorylation of dynactin and each partially rescued tubulation of mutant NPC1 membranes. Evaluating the impact of increased tubulation on cholesterol and GM1 efflux in mutant NPC1 cell lines, improved TVM formation resulted in reduced filipin labeling and redistribution of GM1 out of late endosomes and lysosomes. These findings support the hypothesis that NPC1 TVMs play an important role on cholesterol transport and that loss of TVM formation is linked to defective cholesterol efflux. They also suggest that enhancing TVM formation could be a therapeutic target for NPC disease.

1676/B55
Mutations in Inositol Polyphosphate-5-Phosphatase E Link Phosphoinositide Signaling to the Ciliopathies.
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Phosphatidylinositol phosphates (PtdIns) are a family of derivatives of the membrane lipid phosphoinositides, which are highly concentrated in distinct cellular pools and mediate a number of critical signaling functions such as cell proliferation, apoptosis, metabolism and motility. PtdIns signaling is tightly regulated, both spatially and temporally, by subcellularly localized PtdIns kinases and phosphatases that dynamically alter their activity. Joubert Syndrome, a member of the newly emerging ciliopathies, is a recessively inherited developmental brain disorder characterized by cerebellar midline hypoplasia, retinal dystrophy, nephronophthisis and liver fibrosis. The signaling pathways orchestrated by the primary cilia that lead to this class of diseases are largely unknown. In patients affected with JS, we identified mutations in the INPP5E gene, encoding inositol polyphosphate-5-phosphatase E, which hydrolyzes the 5-phosphate of PtdIns(3,4,5)P3 and PtdIns(4,5)P2. Mutations cluster in the phosphatase domain and impair 5-phosphatase activity, resulting in altered cellular ratios of phosphoinositides, as well as hyperactivation of downstream Akt signaling. INPP5E localizes to cilia in major organs affected in JS, and mutations promote premature destabilization of cilia in response to stimulation. Thus, this data links PtdIns signaling to the cilia, a cellular structure that is becoming increasingly appreciated for its role in mediating cell signals, and cerebellar development.

1677/B56
Myosin 10 Expression in a CNS Stem Cell Model of Prion Disease.
Prion diseases arise from the templated misfolding of normal cellular prion protein (PrP\textsuperscript{C}) into disease specific conformations (PrP\textsuperscript{Sc}). Brain pathology includes characteristic spongiform degeneration, PrP\textsuperscript{Sc} aggregation, PrP\textsuperscript{Sc} accumulation, astrocytic gliosis, and neuronal loss. The specific molecular mechanisms of prion replication and trafficking are not well understood. PrP\textsuperscript{C} may be involved in formation of junctional complexes, cellular adhesions, filopodial extension, and PrP\textsuperscript{Sc} may also be transported between cells using thin intercellular protrusions called tunneling nanotubes, though the method of transport is still unknown. Because of the involvement of the unconventional myosin 10 (Myo10) in filopodial extension, we investigated the expression and localization of Myo10 in normal and prion infected CNS stem cell containing neurosphere cultures. Myo10 localized to filopodia and thin actin based intercellular projections as did PrP\textsuperscript{C} and PrP\textsuperscript{Sc}. The role, if any, of Myo10 in intercellular prion trafficking is being examined in detail.

**1678/B57**

**Heat Shock 70kDa Protein 1A in a Cellular Model of Spinocerebellar Ataxia Type 6.**

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SCA6 is a neurodegenerative disease caused by a polyglutamine (polyQ) expansion in the C-terminus of the Cav2.1 calcium channel (Cav2.1CT). Recent studies suggest that Cav2.1CT exerts toxic effects to cells and that this toxicity may underlie pathogenesis of SCA6. In the present study we characterized the toxicity exerted by Cav2.1CT using cellular models: L24 and S13 cells expressing Cav2.1CT carrying 24 polyQ (disease range) and 13 polyQ (normal range), respectively. When treated with some toxic stimuli such as CdCl\textsubscript{2}, L24 cells showed lower viability than S13 cells and caspase-dependent apoptosis pathway was found to be more activated in L24 cells. Moreover, promyelocytic leukemia nuclear bodies in L24 cells were disrupted, and Cav2.1CT aggregations were bigger than those in S13 cells. Microarray analyses and RNAi experiments revealed that down-regulation of HSPA1A determined the vulnerability of L24 cells after CdCl\textsubscript{2}-treatment. Then we investigated the protein expression of HSF1, because heat shock transcription factor 1 (HSF1) is known to modulate HSPA1A expression. We found that under normal and cadmium-treated conditions, the expression level of HSF1 in L24 cells is lower than that in S13 cells. Interestingly, however, heat shock enhanced the expression of HSF1 at almost the same extent in both L24 and S13 cells. Therefore we tested the effect of heat shock on cell viability of L24 cells after cadmium exposure. We found viability of L24 cells was improved and the number of cells that underwent caspase-dependent apoptosis was decreased. These results strongly suggest that HSF1-HSPA1A axis determines cell death vulnerability in L24 cells.

**Other Diseases (1679 – 1703)**

**1679/B58**

**Cellular Organelle Disease Genomics: Retention of Mutated Proteins in the Endoplasmic Reticulum Is a Common Mechanism of Numerous Human Monogenic Diseases.**

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More than a third of all cellular proteins are targeted to the endoplasmic reticulum (ER) as a first step in their trafficking journeys along the secretory pathway to their final destinations. within the ER lumen, nascent proteins enter into folding pathways assisted by the large number of molecular chaperones present in this organelle. The processes of protein folding, assembly into multi-subunit complexes and export out of the ER are subjected to a stringent quality control system to ensure that only properly folded and assembled proteins are transported out of the ER. Malfolded proteins and orphan subunits of protein complexes are rejected by the ER quality control machinery and as a result are re-translocated to the cytosol for degradation by the
ubiquitin/proteasome systems. The processes of ER retention, re-translocation from the ER to cytosol and degradation by the proteasome have been named ERAD (ER-Associated protein Degradation). ERAD has been implicated in the cellular mechanisms of at least 40 loss-of-function monogenic diseases in human including cystic fibrosis and emphysema. Due to the high stringency of the ERAD and the large number of cellular proteins that has to pass through the secretory pathway (~8,000), we reasoned that ERAD should be implicated in the mechanisms of many more human monogenic diseases. We therefore, utilized bioinformatics and data mining approaches and found that at least 45% of all known human disease genes have an ER-targeting signal and we identified many ERAD disease candidates. This is significantly higher than the proportion of the cellular proteins with ER targeting signals (30%) and may indicate that the excessive stringency of ERAD is contributing to human pathology. Furthermore, we validated our predictive approach experimentally by establishing that ERAD is indeed responsible for most of the loss-of-function missense mutations in the genes involved in Robinow syndrome, Acromesomelic Dysplasia type Maroteaux and Familial Hypercholesterolemia.

1680/B59
17β Estradiol (E2) Inhibits STIM1-Dependent Ca²⁺ Influx in Airway Epithelia.
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STIM1 acts as a Ca²⁺ sensor and the lowering of endoplasmic reticulum (ER) Ca²⁺ initiates the translocation of STIM1 from the ER to regions close to the plasma membrane, activating Ca²⁺ influx. We have previously shown that E2 inhibits ATP-stimulated Ca²⁺ signaling in airway epithelia and we propose that this inhibition contributes to the gender difference seen in cystic fibrosis lung disease(1). To better understand this process, we tested the hypothesis that E2 impairs STIM1 function, thereby limiting Ca²⁺ influx. We found that STIM1 is highly expressed in airway epithelia and nocodazole pre-treatment to disrupt microtubules abolished 10 μM ATP-stimulated Ca²⁺ influx (n=6), as measured using standard Fura2 techniques in airway epithelia, suggesting that STIM1 migration along microtubules is required to initiate Ca²⁺ influx in airway epithelia (n=6). siRNA knockdown of STIM1 (<90%) abolished ATP-stimulated Ca²⁺ influx (n=6). In contrast, a control siRNA (directed against γENaC) was without effect (n=6). Importantly, 10 nM E2 had no affect on basal or ATP-dependent Ca²⁺ signaling once STIM1 had been knocked down (n=6). Overexpression of either cfpSTIM1 or yfpSTIM1 resulted in a constitutively active Ca²⁺ influx pathway that was nocodazole-sensitive (both n=10). We next tested whether STIM1 aggregation was affected by E2 using acceptor photobleaching FRET of CFP and YFP-tagged STIM1 constructs co-expressed in airway epithelia. Under basal conditions there was 10±1.2% FRET, which was significantly increased to 22±2.7% after 10 μM ATP exposure (n=9). The increase in FRET was most noticeable around the peri-nuclear region. Pretreatment with nocodazole reduced basal FRET by 30% and prevented the increase in FRET with ATP (n=10), suggesting that fluorescently-labeled STIM1 functions similarly to native STIM1. 10 min E2 pre-exposure had no affect on basal STIM1 FRET (9.8±0.9%; n=12). However, E2 prevented the ATP-dependent increase in FRET (post E2/ATP FRET was 9.6±1.3%; n=12). Thus, we conclude that E2 acts non-genomically in airway epithelia by inhibiting STIM1 function, thereby preventing Ca²⁺ influx. (1) Coakley R et al., JCI, 118(12):4025-35. 2008.

1681/B60
Up-regulation of ENaC Expression and Function by an ER Molecular Chaperone Calreticulin.
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Epithelial sodium channel (ENaC) is a heteromultimeric Na⁺ channel at the apical membrane in the kidney, colon, and lung. In these organs, ENaC plays a crucial role in regulating Na⁺

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absorption and extracellular fluid volume. The significance of ENaC in fluid volume control was further highlighted by recent studies on β-ENaC transgenic mice. The mice with airway-specific overexpression of β-ENaC show defective mucus transport and decreased survival after birth. Because of its vital functions in Na⁺ transport and homeostasis, it is important to understand the molecular mechanisms of ENaC regulation including its expression, synthesis and intracellular trafficking. Despite the importance of ENaC, its protein quality control mechanism remains less established. Here we firstly show the role of calreticulin (CRT), a lectin-like molecular chaperone in the endoplasmic reticulum (ER), on the regulation of ENaC. Overexpression and knockdown analyses clearly indicated that CRT positively affects the expression of each ENaC subunit (α, β and γ). CRT overexpression also up-regulated the cell surface expression of α-, β- and γ-ENaC. Moreover, we found that CRT directly interacts with each ENaC subunit. Although CRT knockdown did not affect the de novo synthesis of ENaC subunits, CRT overexpression decreased α-, β- and γ-ENaC expression in the detergent (RIPA)-insoluble fraction, suggesting that CRT enhanced the solubility of ENaC subunits. Consistent with the increased intracellular and cell surface expression of ENaC subunits, increased channel activity of ENaC was also observed upon overexpression of CRT. Our study thus identifies CRT as an ER chaperone that regulates ENaC expression and function.

1682/B61 Neutral Sphingomyelinase 2 Is a Phosphoprotein Activated by Oxidative Stress via Down Regulation of Calcineurin Phosphatase.
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We previously reported that human airway epithelial (HAE) cells exposed to H2O2- or to cigarette smoke-oxidative stress accumulate ceramide and initiate apoptosis, via sphingomyelin hydrolysis, specifically exerted by over activation of the redox sensitive neutral sphingomyelinase 2 (nSMase2). Here we show by In Vivo labeling with 32P orthophosphate that nSMase2 is a phosphoprotein and identify by mass spectrometry analysis 5 phosphoserine residues. We show that the level of phosphorylation is affected by both Anisomycin and phorbol-12-myristate-13-acetate (PMA/TPA) treatment, thus suggesting that P38 MAPK and PKC(s) may be upstream of nSMase2 constitutive phosphorylation. Treatment of HAE cells for 30' with 250µM H2O2 enhances nSMase2 phosphorylation about 1.5 fold. We generated a full phosphoserine-alanine mutant (MT) and demonstrated that although the MT is still active the inducible activity with H2O2 is abolished. We then used yeast-2-hybrid with human nSMase2 as a bait to interrogate a cDNA library, searching for candidates that physically interact with nSMase2. Calcineurin phosphatase (CaN) was identified as such a candidate. Moreover, co-immunoprecipitation analysis confirmed that CaN indeed interacts with nSMase2, but not under H2O2-induced oxidative stress. In addition, a mutant of nSMase2 that lacks the binding site of CaN was found to be constitutively over phosphorylated and active in comparison to the WT. Since CaN does not bind nSMase2 under oxidative stress, we suggest that H2O2 enhances nSMase2 activity via down modulation of the regulative role of CaN, thus allowing nSMase2 to be fully phosphorylated and activated downstream of p38 MAPK and PKC(s).

1683/B62 Induction of Heat Shock Proteins in the Livers of C57BL/6 Mice Exposed to Asian Dust.
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Heat shock proteins (HSPs), which function mainly as molecular chaperones, are induced in response to a variety of stressful situations including environmental, physical and chemical stimuli. The induction of HSPs helps to limit the damage and facilitate cell recovery, and thus it is regarded as important in maintaining cellular homeostasis under the conditions of stress. Moreover, as some members of the HSP family, such as hsp60 and hsp90, were reported to act as danger signaling molecules by activating the cells of the innate immune system directly, there has been considerable interest in HSPs. Here, we investigated whether inhaled Asian dust (AD),
which is a seasonal phenomenon in the East Asian countries and generally composed of the particulate matter 10 (PM10) and the ultrafine particles, could induce hsp60 or hsp90 expression in murine liver. for this study, C57BL/6 mice were injected with different doses (20 μl of 0.1, 0.2 or 0.4 mg/ml), and times (three times a week for 4, 8 or 12 weeks) of AD particles. Liver specimens were obtained 24h after intratracheal instillation. Detection of hsp60 or hsp90 expression was determined by western blotting analysis using specific antibodies. Our results indicate that levels of hsp60 or hsp90 expression were elevated in the livers of mice injected with AD. In this study, therefore, we examined the induction of HSPs, especially in response to exposure to AD particles.

1684/B63  
Connective Tissue Growth Factor Localizes to Activated Pancreatic Stellate Cells in a Model of Ethanol-induced Pancreatic Fibrosis.  
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BACKGROUND: Excessive alcohol consumption in humans is frequently associated with chronic pancreatitis which is characterized by cell death (necrosis), inflammation, and scarring, the latter of which arises due to deposition of collagen by pancreatic stellate cells (PSC), a relatively minor cell population that transitions to an activated alpha-smooth muscle actin (α-SMA)-positive phenotype after tissue injury. Studies of activated PSC In Vitro have shown that they produce connective tissue growth factor (CTGF), a highly pro-fibrogenic molecule. OBJECTIVES: to establish a model of ethanol-induced pancreatic fibrosis in mice (a species that is highly tolerant to the toxic effects of ethanol) and to identify CTGF-producing cells in fibrotic regions in vivo.

METHODS: Six-week old C57Bl/6 male mice were injected i.p. with 3.2 g ethanol/kg once per day, six days per week, for three weeks. on one day each week, some mice also received i.p. injections of cerulein (a cholecystokinin analog; 50 μg/kg body weight) each hour for 6 hours.

RESULTS: Peak blood alcohol levels were 400mg/dl in ethanol-treated mice and 31mg/dl in mice receiving water (P<.001). In mice receiving ethanol plus cerulein, there was increased frequency of α-SMA-positive cells or collagen deposition as compared to mice receiving either cerulein alone or ethanol alone (P<.001). In mice receiving either ethanol alone or both ethanol and cerulein, collagen, CTGF, and α-SMA were co-localized to the same cells, which were located in areas of intense fibrosis and were often adjacent to areas of necrosis. Furthermore, collagen Iα1, CTGF, and α-SMA mRNA also co-localized in the same cells as assessed by in situ hybridization.

CONCLUSIONS: Our data show that (i) the model of alcohol-induced chronic pancreatitis in mice is rapid, efficient, and mimics key pathophysiological features of the disease in humans; (ii) activated PSC are intimately associated with the production of α-SMA, collagen, and CTGF during alcohol-induced pancreatic injury and (iii) In Vivo production of CTGF by activated PSC may have important implications for novel therapeutic strategies to treat or prevent pancreatic fibrosis.

1685/B64  
Responses of Pro- and Anti-angiogenic Factors to Capillary in Rat Soleus Muscle after Hindlimb Unloading.  
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PURPOSE: A coupling between decreased muscle use and reduced muscle capillarity was noted. Muscle atrophy associated with capillary regression involved at least two independent mechanisms: one is specific angiogenic factors, such as vascular endothelial growth factor (VEGF) and angiopoietin, and the other is thrombospondin-1 (TSP-1) that inhibits angiogenesis by inducing apoptosis in endothelial cells. The aim of the present study was to determine the
responses of pro- and anti-angiogenic factors to capillary in rat soleus muscle after hindlimb unloading (HU). METHODS: The hindlimbs of male Wistar rats were unloaded by tail suspension and then the soleus muscles were isolated after 2 weeks of HU. To clarify the three-dimensional architecture of the capillary network, contrast medium-injected rat soleus muscles were visualized clearly using a confocal laser scanning microscope. Apoptotic endothelial cells were identified by DNA fragmentation using immunofluorescent staining. In the second reaction, these sections stained with TUNEL reagents were incubated with anti-von Willebrand factor antibody to identify endothelial cells. Furthermore, VEGF, KDR, Flt-1, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), Tie-2, hypoxia inducible factor-1alpha (HIF-1alpha), and TSP-1 mRNAs were determined by TaqMan probe-based real-time PCR reactions. RESULTS: The numbers of anastomoses and the tortuosity were significantly lower in the atrophied muscle, suggesting that capillary regression appears to occur. VEGF, Ang-1, their receptors, and HIF-1alpha mRNA expressions decreased significantly in the atrophied muscle. No significant change was observed in the expression of Ang-2. As a result, the Ang-2-to-Ang-1 ratio increased, suggesting that the vasculature destabilizes. Ang-2 expression in the absence of VEGF caused vessel regression. TSP-1 expression increased in the atrophied muscle. Furthermore, HU caused capillary regression with vascular endothelial cell apoptosis. CONCLUSION: The present study provides experimental evidences that both decreased angiogenic factors and increased TSP-1 expression may help to explain the reduced capillarity in the atrophied muscle.

1686/B65

Activation of the Raf-1/Mek/Erk Signaling Pathway by Thyroid Hormone Is Mediated by Thyroid Hormone Receptor Beta in Endothelial Cells.
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Several diseases are caused by either the increase (cancer) or decrease (cardiovascular diseases) of blood vessels. Proper regulation of the formation of new blood vessels (angiogenesis) is important in the treatment development of these pathological states. The angiogenic effect of thyroid hormone has been described however, the mechanism remains unknown. A direct connection between cell growth, cell proliferation and angiogenesis in endothelial cells exists. Protein synthesis is essential for cell proliferation, and the activation of ERK by phosphorylation in the mitogen-activated protein kinase (MAPK) pathway is known to directly link growth factor signaling and ribosome synthesis. We hypothesized that thyroid hormone (T3) modulates the activity of the MAPK cascade by activation of specific thyroid hormone receptors (TRs). Therefore, we investigated the role of TR-alpha and TR-beta on the activation of the MAPK pathway in endothelial cells in culture. Cells cultured in T3 free media were incubated with T3 (10nM and 100nM) for 48h. MAPK activation was assessed by analyzing the phosphorylated and non-phosphorylated forms of RAF-1, MEK and ERK proteins using Western blot. Results showed that T3 at 10nM and 100nM increased phosphorylated RAF-1, MEK and ERK proteins. In contrast, T3 at 1nM had no effect. To determine which isoform TR was responsible for the activation of the MAPK pathway, endothelial cells were infected with adenovirus carrying TR-alpha or TR-beta transgenes inducing over-expression of these receptors. Cells over-expressing TR-beta presented considerably increase in phosphorylated ERK. In conclusion, thyroid hormone activates the MAPK pathway in a dose dependent fashion. Moreover, there is evidence that TR-beta mediates this activation.

1687/B66

Beer Polyphenols Regulate Angiogenic and Inflammation Processes: In Vivo Results.
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Despite their distinct etiopathogenesis, cardiovascular disease, cancer, diabetes and obesity are being considered angiogenesis-dependent diseases, associated with a chronic inflammation state and oxidative stress that together with hypoxia are the main stimulus of the angiogenic process.
originating unstable and leaky vessels. Hop-derived supplements and beer contain several polyphenols: xanthohumol (XN), which can be converted to isoxanthohumol (IXN), and in the potent phytoestrogen 8-prenylnaringenin (8PN). Our study aims to confirm In Vivo modulation of angiogenesis and inflammation by XN, IXN, and 8PN. C57BL/6J mice were inoculated subcutaneously with Matrigel® containing vascular endothelial growth factor (VEGF) with or without polyphenols. After 7 days, plugs were removed and hemoglobin content and serum N-acetylglucosaminidase (NAG) activity were measured. for skin wound-healing assay, full skin-thickness longitudinal incisions were created on the dorsal skin of Wistar rats. Polyphenols were administered topically during 7 days. Wounded tissue was collected for histology and inflammatory markers in the serum were measured. Student’s t-test or ANOVA followed by the Bonferroni test were used for statistical analysis. Differences were considered significant whenever p< 0.05. Both plug and skin wound-healing assays confirmed that treatments with XN and IXN reduced vessels number assembly and decreased serum NAG activity, while 8PN increased the formation of blood vessels in both assays and enzyme activity in the wound-healing assay. A similar profile was found for serum interleukin (IL)1β determination in the wound healing assay. Our results demonstrate that while 8PN stimulates angiogenesis, XN and IXN manifested anti-angiogenic and anti-inflammatory effects. These findings suggest that the effects observed on vascular wall cells must be carefully taken into account as these polyphenols belong to the same metabolic pathway. Furthermore, these results may provide clues to the development of useful therapeutic agents against inflammation- and angiogenesis-associated pathologies. Supported by FCT (SFRH BD/41888/2007), iBeSa (P10-08) and University of Porto/Santander Totta.

1688/B67
Novel Molecular Signatures for Human Pulmonary Arterial Hypertension.
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Objective: Many studies have attempted to understand the clinical and pathological conditions of pulmonary arterial hypertension (PAH); yet, it remains an incurable disease, in need of less invasive diagnostic tools and a better patient-specific therapy. Methods: we analyzed the plasma protein profile of 51 healthy volunteers and 113 PAH patients, 58 idiopathic (IPAH), 55 non-idiopathic (NIPAH), using multi-analyte profiling (MAP) of a commercial antigen array. Results: Analysis of 100 μl of patient plasma identified 63 antigens whose levels were significantly (p<0.05) increased (n=53) or decreased (n=10) in PAH patients when compared to controls. These novel disease-associated antigens include: onco-fetal proteins, growth factors, pro-angiogenic molecules, stem cell factors, immune-inflammatory mediators, and components of the extracellular matrix. Importantly, 22 antigens were significantly increased or decreased between IPAH and NIPAH patients, potentially allowing for discrimination of different forms of the disease. Of note, significant differences were identified in the levels of erythropoietin (EPO) in PAH patients, a known pro-angiogenic, anti-apoptotic molecule. To test the bioactivity of EPO in PAH patient samples lung endothelial cell (EC) angiogenesis assays were performed in which PAH-patient-derived EPO was shown to promote network formation when compared to controls. In addition, pulmonary artery smooth muscle cell proliferation was increased with EPO stimulation compared to controls. In keeping with this In Vitro finding, EPO levels were shown to be increased in ECs undergoing neo-angiogenesis during pulmonary vascular remodeling associated in PAH human lung tissue sections. Conclusions: Collectively, these novel results provide new insights into the pathophysiology and potential diagnosis of PAH.

1689/B68
ERBB Receptor Activation Is Required for Pro-fibrotic Transforming Growth Factor β Signaling.
Engagement of the transforming growth factor β (TGF-β) receptor complex activates multiple signaling pathways that play crucial roles in both health and disease. Deciphering the molecular basis for these pathways will help address fundamental issues pertaining to TGF-β biology and may offer unique opportunities for therapeutic intervention. Previous studies have established the importance of TGF-β as a key regulator in fibrogenesis and cancer-associated desmoplasia. However, its exact mode of action in these pathological processes has remained poorly defined. Here we report a novel mechanism whereby signaling via ERBB receptors serves as a central requirement for the pro-fibrotic activity of TGF-β. We show that TGF-β triggers upregulation of ERBB ligands and activation of cognate receptors via the canonical SMAD pathway, primarily SMAD3, in fibroblast cells. Interestingly, these events are commonly observed in a subset of fibroblast but not epithelial cells from different species, indicating cell-type specificity. Moreover, using genetic and pharmacological approaches, we demonstrate that ERBB activation by TGF-β is essential for the induction of fibroblast cell anchorage-independent growth and contributes to extracellular matrix production. Likewise, the TGF-β-driven stimulation of fibroblast cell motility and morphologic transformation are modulated by ERBB receptors. Together, these results uncover important aspects of TGF-β signaling that highlight the role of ERBB receptors as critical mediators in fibroblast responses to this pleiotropic cytokine.

1690/B69
ApoF and ApoE as Candidate Effecters of Cholesterol Efflux through the Basal Plasma Membrane of Lacrimal Acinar Cells.
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Objective: Our previous studies have suggested that Apolipoprotein F (ApoF) and Apolipoprotein E (ApoE) may participate in cholesterol efflux in lacrimal gland (LG) acinar cells. The purpose of this study was to further investigate the role of these proteins in cholesterol efflux process. Methods: The cellular localization of ApoF and ApoE were analyzed by confocal fluorescence microscopy, while lipid content was assessed by Oil Red O staining with LG sections from up to 24-week male NOD, BALB/c, C57BL/6 and ApoE null mice. Results: The basolateral membrane region of acinar cells from all different strains except the ApoE null mouse showed abundant ApoE labeling. As detected previously, ApoF was enriched at the basolateral membrane of the acinar cells from BALB/c mice but was redistributed to the sub-apical and apical regions from NOD mice. Additionally, ApoF was concentrated in the sub-apical and apical regions of the acinar cells from both wild-type C57BL/6 and ApoE null mice. Oil Red O staining showed that lipid deposition was initiated in the acinar cells from C57BL/6 and ApoE null mice at a younger age than in BALB/c mice although at an older age than in NOD mice. Inactivation of the ApoE gene accelerated the onset of lipid accumulation by 2 weeks while increasing its severity compared to the parent C57BL/6 strain. The basolateral enrichments of ApoE and ApoF were highly colocalized with actin filaments in patchy and extended tubular structures. Conclusions: Reduction in basolateral concentration of ApoF and inactivation of ApoE are correlated with lipid deposition in LG acinar cells, suggesting that these proteins may regulate cholesterol efflux in peripheral cells which actively produce and consume cholesterol. The colocalization of ApoF and ApoE with actin in patches is suggestive of these events occurring on lipid rafts at the basolateral plasma membrane. Support: EY011386

1691/B70
VEGF-R3, Cognate Receptor for VEGF-C and D as Potential Therapeutic Target for Lymphangioleiomyomatosis.
Lymphangioleiomyomatosis (LAM) is a potentially fatal lung disease characterized by nodules of proliferative smooth muscle-like cells formed around lymphatic vessels and expressing VEGF-R3. Since VEGF-R3 is the cognate receptor for VEGF-C and D, we hypothesized that LAM cell proliferation might be induced by these growth factors through activation of VEGF-R3. Using primary human LAM derived smooth muscle-like cells (LDC) we found for the first time that VEGF-C or D induced a dose-dependent proliferation of LAM cells. This proliferative response was blocked by the indoline compound MAZ-51 which specifically inhibits the tyrosine kinase activity of VEGF-R3. Western Blot of immunoprecipitated VEGF-R3 from LDC lysates showed that in the presence of VEGF-C or D, VEGF-R3 is phosphorylated and that VEGF-R3 inhibitor blocks this activation. In contrast, normal primary Pulmonary Artery SM (PASM) cells showed no expression of VEGF-R3 and appropriately no receptor phosphorylation when stimulated with VEGF-C. These findings confirm that VEGF-C and D induced LDC proliferation is at least, in part, a result of activation of their cognate receptor, VEGF-R3. LCD’s were also found to adhere and migrate on VEGF-C or D matrices. We then characterized the downstream signaling intermediates mediating VEGF-R3’s proliferative effect and found that it was mediated by activation of a pathway involving PI3K/Akt/mTOR and subsequently S6. Furthermore, we found that activation of LDC was autocrine in nature through release of VEGF-C following interactions with lymphatic endothelial cells. Taken together these results suggest a novel pathogenic mechanism for LAM through the lymphangiogenic proteins VEGF-C and D and identify VEGF-R3 as a novel potential pharmacotherapeutic target.

1692/B71
Water Soluble Wolfberry Phytochemicals Have Increased Antioxidant Activity.
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Objective: Consistent consumption of fruits and vegetables is associated with reduced risk of developing chronic diseases, at least partially due to the antioxidant capacity. Wolfberry (Lycium barbarum) is a food type of fruits which has been consumed for decades. The current study is to investigate the protective effect of water soluble phytochemicals of wolfberry on cellular oxidative stress damage. Methods: The cell lines used in this study were human retinal pigment epithelial ARPE19, mouse neuronal HT22, and rat neuronal PC12. Wolfberry fruits were purchased from the local grocery store. The water soluble wolfberry phytochemicals were extracted, freeze dried, and redissolved in PBS or cell culture medium. Antioxidant activity was determined by ferric-reducing antioxidant power (FRAP) assay. H2O2 was added to cell culture medium to induce oxidative stress. In addition, ARPE19 cells were also exposed to high glucose for 60 hrs to mimic hyperglycemic oxidative stress. Cell viability was detected by MTT assay. Western blotting was used to monitor alteration of protein expression. Results: In Vitro FRAP assay results showed that water soluble wolfberry phytochemicals had very high antioxidant activity (7771.2 μM Trolox equivalent per 100 grams). H2O2 at 100 μM for 24 hrs significantly inhibited cell viability of the three cell lines. High glucose challenge at 36 mM for 60 hrs dramatically decreased cell viability. Application of water soluble wolfberry phytochemicals at 1 mg/mL restored cells from H2O2 and/or hyperglycemia-induced oxidative stress. Mechanism studies further demonstrated that wolfberry phytochemicals scavenged cellular reactive oxygen species, suppressed NF-κB, and restored AMPK. Conclusions: Water soluble wolfberry phytochemicals act as reactive oxygen species scavengers which, in turn, protect cells from oxidative stress. The results suggest that the water soluble wolfberry phytochemicals may be used to develop novel complementary therapeutic agents and/or dietary regimens for prevention of chronic diseases.
1693/B72

**Bicarbonate-Secreting NBCe1 and CFTR Expressing Villus Enterocytes Function to Expand the Condensed Mucus Secreted By NKCC1 Expressing Goblet Cells.**

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Intestinal disorders are linked to impaired anion transport and abnormal mucus. We tested the hypothesis that the generation of condensed acidic mucus and its transformation to expanded alkaline mucus involves membrane trafficking of distinct, cell-specific anion transporters.

**Methods:** Ligated duodenal, jejunal, and ileal segments were left intact, or treated with 8-Br-cAMP (pH 7.4; 100 μM); carbachol (pH 7.4; 10 μM); acid (HCl-saline, pH 2.0) or bicarbonate (HCO3- saline; pH 8.0, 8.5, or 9.0). The effects of treatments were evaluated on (1) mucus expansion, (2) luminal pH, and (3) functional expression of the anion transporters Na”K’2Cl’ (NKCC1), Na’HCO3’ (NBCe1) and CFTR. **Results:** All untreated villus enterocytes expressed low levels of NKCC1, NBCe1 and CFTR. NKCC1 was observed in intracellular endosomes while NBCe1 and CFTR were partially membrane bound. All goblet cells expressed NKCC1, that was partially membrane bound, but no NBCe1 or CFTR was detected. NKCC1 protein levels correlated with previously reported data on goblet cell abundance and condensed acidic mucus layer thicknesses along the intestinal tract. Luminal pH in untreated duodenum, jejunum and ileum was alkaline (pH 7.5 to 9.0) and correlated with the thickness ratios of expanded/condensed mucus. Bicarbonate induced robust mucus expansion in all segments. Other treatments were ineffective. cAMP and carbachol (20 min) reduced luminal pH, induced up-regulation and membrane-recruitment of NBCe1, NKCC1 and CFTR in enterocytes and NKCC1 in goblet cells. After 45 min, carbachol induced volume loss, and transporter internalization to endosomes. Acid or HCO3- treatments produced similar tissue responses: up-regulation and membrane-recruitment of NBCe1 and CFTR in enterocytes and NKCC1 in goblet cells. But NKCC1 was completely internalized in enterocytes. **Conclusions:** NKCC1 expressing goblet cells secrete HCO3- free fluid and produce condensed acidic mucus. Luminal HCO3’ + H’ (in condensed mucus) or luminal H’ + HCO3’ (in expanded mucus) both generate CO2. In enterocytes, the intracellular pH reducing effect of CO2 inhibits NKCC1 by internalization, but activates HCO3- secretion by membrane recruitment of NBCe1 and CFTR.

1694/B73

**Cell Therapy of Corneal Diseases with Umbilical Mesenchymal Stem Cells.**

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To develop a cell therapy strategy for corneal diseases, human umbilical mesenchymal stem cells (HUMSC) were transplanted into corneas of lumican knockout (Lum-/-) mice via intrastromal injection. Lumican is a keratansulfate proteoglycan and belongs to the small leucine rich proteoglycan (SLRP) family in corneal stroma, and has a pivotal role in the maintenance of corneal transparency. Lum-/- mice manifested thin and cloudy corneas due to the absence of lumican causing the disorganization of extracellular collagenous matrix in stroma. Transplantation of HUMSC significantly improved corneal transparency and increased corneal stromal thickness in Lum-/- mice, whereas transplantation of human umbilical hematopoietic stem cells failed to do so. Histological and immune fluorescence staining revealed that HUMSC survived in mouse corneal stroma for a prolonged period of time (>3 months), and could trans-differentiate and assume keratocyte phenotypes, e.g., dendritic morphology, expression of keratocytes markers such as keratocan, aldehyde dehydrogenase, CD34 with little or no graft rejection reactions. In contrast, transplanted HUHSC rapidly vanished from the mouse corneas due to graft rejection as characterized by the presence of CD45, CD90, and F4/80-positive cells. Our results suggest that transplantation of umbilical mesenchymal stem cells is a potential treatment regimen of congenital and/or acquired corneal diseases.
1695/B74
Chronic Arsenic Exposure at Municipal Drinking Water Levels Downregulates P2 Receptor-Dependent Ca²⁺ Signaling and Wound Repair in Airway Epithelial Cells.
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Arsenic is a natural toxicant that is tightly controlled in drinking water supplies with a current maximum containment level of 10 parts per billion (ppb). Arsenic is found in the lung after ingestion and can alter lung tissue and function. While chronic exposure to moderate and/or high levels of arsenic in drinking water may lead to the development of disease in humans, the effects at low-dose are inferred mostly through models of high-dose exposure. We have shown that chronic exposure to low-dose arsenic (<50 ppb) in animal models increases lung biomarkers that indicate altered wound repair and, acute low-dose arsenic (15-300 ppb) inhibits wound closure in human airway epithelial cells (16HBE14o-). Calcium ion (Ca²⁺) is an important molecular messenger associated with many of the basic functions of the lung epithelium, including wound repair. Significantly, localized wounds lead to increased extracellular ATP that results in a coordinated increase in [Ca²⁺]. In this report, we employ a chronic In Vitro model to more closely evaluate the effects of low-dose arsenic exposure on airway epithelial cells. 16HBE14o- cells were exposed to 0, 10, or 25 ppb arsenic through several passages and evaluated for purinergic and localized wound responses. A significant reduction in Ca²⁺ response to ATP (500 nM or 1 μM) was observed in cells exposed to either 10 or 25 ppb arsenic. Further, coordinated [Ca²⁺] changes following a localized cell wound were downregulated in a dose-dependent manner (0, 10, 50 ppb). Using real-time RT-PCR, we found a significant loss of purinergic receptor P2Y₂ expression in response to chronic arsenic exposure, with no changes in P2Y₄ or P2X₄. In order to examine Ca²⁺ signaling under conditions of natural exposure, we cultured mouse tracheal epithelial (MTE) cells from control and chronically arsenic-exposed mice via drinking water. Despite culture in arsenic-free conditions, MTE cells from arsenic-exposed mice (50 ppb) had a significantly reduced response to ATP (1 μM). We conclude that low-level arsenic exposure reduces lung epithelial cell Ca²⁺ signaling and this signaling likely contributes to altered lung wound repair as well as other basic functions of the airway epithelium.

1696/B75
Interaction of KRIT-1 (CCM1) and CCM2 (Malcavernin, OSM) Regulates Vascular Barrier Function by Inhibiting Rho-Kinase.
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Endothelial cell-cell junctions regulate vascular permeability, vasculogenesis and angiogenesis. Familial Cerebral Cavernous Malformations (CCM) result from heterozygous null mutations of Ccm2 (Malcavernin, OSM), Ccm3 (PCDC10), or Krit1 (CCM1), a Rap-1 effector that stabilizes endothelial cell-cell junctions. Homozygous deletions of Krit1 or CCM2 produce lethal vascular phenotypes in mice and zebrafish. Here we report physical interaction of KRIT-1 and CCM2 proteins is required for endothelial cell-cell junctional localization of both, and deficit of either protein destabilizes barrier function by sustaining activity of RhoA and its effector, Rho kinase (ROCK). Protein-haploinsufficient Krit1+/- or Ccm2+/- mice exhibited increased endothelial permeability In Vitro and vascular leak in vivo, reversible by fasudil, a ROCK inhibitor. Furthermore, ROCK was hyperactive in sporadic and familial human CCM endothelium as judged by increased phosphorylation of myosin light chain. These data establish that Krit1-CCM2 interaction regulates vascular barrier function by suppressing ROCK signaling, that this pathway is dysregulated in human CCM endothelium, and indicate that fasudil could ameliorate both CCM disease and vascular leak.
1697/B76
Ectopic Expression of Connexin 31 Results in EKV-like Skin Abnormality in Mouse.
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Connexins form gap junctional channels between adjacent cells in various tissues including epidermis. Mutations in connexin 31 (Cx31) are associated with hearing impairment, erythrokeratodermia variabilis (EKV), and peripheral neuropathy. The pathophysiological function of Cx31 in epidermis remains largely unknown. In the present study, we have ectopically expressed human Cx31 in mouse skin under the control of mouse K14 promoter. The resulted transgenic mice show impaired wound healing and hyperkeratosis. Further analysis reveals that overexpression of Cx31 leads to hyperproliferation, parakeratotic hyperkeratosis, and abnormal differentiation of keratinocytes. These phenotypes resemble the pathological features of EKV in human. At molecular level, exogenous Cx31 induces expression of and interaction with endogenous Cx26. Increased expression of multiple genes involved in cell proliferation and differentiation, including c-Fos, JunB, cytokeratin 6 and cytokeratin 16, is also detected in Cx31 expressing skin samples. The results suggest that Cx31 and Cx26 may coordinate regulation of skin homeostasis through activator protein 1 (AP-1) mediated pathway, providing a novel mechanism for Cx31-associated skin diseases. Meanwhile, the study generates a novel mouse model for human EKV.

1698/B77
Disruption of ZO-1 Localization in the Rabbit Corneal Epithelium In Vivo by Contact Lens-Induced Hypoxia.
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Objective. Hypoxia impairs the barrier function of the corneal epithelium. This function depends on tight junctions, of which zonula occludens (ZO)-1 is a major component. We have now investigated the effects of hypoxia on ZO-1 localization and expression in the rabbit corneal epithelium in vivo. Methods. A polymethylmethacrylate (PMMA) or rigid gas-permeable (RGP) lens was applied to one eye of albino rabbits for 24 hours. The structure of the corneal epithelium was examined by In Vivo confocal microscopy, and epithelial barrier function was evaluated by measurement of central corneal thickness. The distribution and expression of ZO-1 in the corneal epithelium were examined by immunofluorescence analysis and by immunoblot and reverse transcription-polymerase chain reaction analyses, respectively. Results. Application of a PMMA lens, but not that of an RGP lens, resulted in a reduction in cell size at the surface of the corneal epithelium, as well as an increase in central corneal thickness. Immunofluorescence analysis revealed a continuous pattern of ZO-1 immunoreactivity around the perimeter of superficial corneal epithelial cells in control eyes or in eyes treated with an RGP lens. In contrast, the pattern of ZO-1 staining was discontinuous and patchy in eyes treated with a PMMA lens. The amounts of both ZO-1 mRNA and protein in the corneal epithelium were reduced by application of a PMMA lens but not by that of an RGP lens. Conclusions. Hypoxia at the ocular surface induced disruption of tight junctions between superficial cells in the rabbit corneal epithelium in vivo.

1699/B78
Respective Roles of Rictor and Raptor in Cardiac Cell Growth and Survival.
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The mammalian target of rapamycin (mTOR) is an effector of phosphatidylinositol-3-phosphate kinase (PI3K) promoting cell growth by forming an mTOR complex 1 (mTORC1) upon binding of the adaptor protein raptor. However, mTOR also binds the protein rictor, forming an mTORC2
complex that activates the survival kinase Akt. The function of mTORC2 in the heart is unknown. We tested the hypothesis that mTORC2 promotes cardiac cell survival through Akt, while mTORC1 is involved in cell growth, upon PI3K activation. Adenoviruses harboring a sequence for selective RNA silencing (siRNA) of either raptor or rictor were generated and used to infect isolated neonatal cardiac myocytes. PI3K activity was stimulated upon addition of 0.1 microM insulin. Apoptosis was stimulated by addition of 5 microM chelerythrine. Upon addition of the adenovirus, a dose-dependent knockdown of up to 90% was obtained for either raptor or rictor. A dose achieving a 65% inhibition was chosen for subsequent experiments. Insulin did not affect raptor expression but it increased rictor abundance by 3-fold (P<0.05). Rictor but not raptor siRNA abolished Akt phosphorylation on S473, both in basal conditions and upon insulin stimulation. Upon raptor knockdown, protein synthesis, measured by tritiated phenylalanine incorporation, was significantly decreased by 50% in basal conditions (P<0.05), and insulin-induced stimulation of protein synthesis was abolished. Reciprocally, rictor siRNA was without effect on these parameters. Upon addition of chelerythrine, rictor siRNA increased apoptosis, as measured by caspase-3 activity, by 100% (P<0.01), whereas raptor siRNA increased it by only 30% (P<0.05). We therefore conclude that mTORC1 participates in cardiac cell growth whereas mTORC2 controls Akt phosphorylation and is mainly involved in cardiac cell survival.

1700/B79
H11 Kinase/Hsp22 Plays an Essential Role in the Cardiac Stress Response.
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H11 kinase/Hsp22 (H11K) is a heat shock protein expressed mainly in the heart, which is upregulated in conditions of pressure overload and myocardial ischemia, two main forms of cardiac stress. H11K promotes cardiac survival and activates the expression of the inducible NO synthase (iNOS) mediating the delayed window of ischemic preconditioning, the most powerful prophylaxis against myocardial infarction. We tested the hypothesis that H11K is essential for the cardiac response to pressure overload and ischemia, using a knockout (KO) model of Hsp22 deletion and a transgenic (TG) mouse model of Hsp22 cardiac over-expression. In basal conditions, no significant differences were found between KO and wild type (WT) in terms of left ventricular size, myocyte cross-sectional area, wall thickness and contractility, whereas TG mice developed cardiac hypertrophy with normal function. During pressure overload induced by chronic aortic banding, KO mice showed significantly (P<0.05) impaired activation of Akt and ERK1/2 survival signaling pathways, abolished iNOS expression, signs of heart failure (significant increase in filling pressure and decrease in ejection fraction), and doubled mortality. Reciprocally, the TG mouse showed significantly (P<0.05) increased iNOS abundance and reduced infarct size after ischemia. Protection by H11K in TG mice was abolished upon iNOS inhibition with L-NAME. H11K-mediated iNOS expression was regulated by the stress-activated STAT and NF-kappaB pathways. Accordingly, STAT and NF-kappaB signaling was impaired in the KO mouse and stimulated in the TG model. Therefore, Hsp22 is both essential and sufficient for pre-emptive protection of the heart at risk of pressure overload and myocardial ischemia through stimulation of stress-activated signaling pathways.

1701/B80
Targeted Inactivation of KIF5B in Pancreatic β-Cells Leads to Insulin Secretory Deficiency and Increased β-Cell Mass In Vivo.
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Objective: Suppression of Kinesin 1 by antisense oligonucleotides(1), or overexpression of dominant-negative acting Kinesin(2), has been reported to affect the sustained phase of glucose-stimulated insulin secretion in β-cells in vitro. In this study, we examined the In Vivo physiological role of Kif5b (Kinesin 1) in β-cell development and function. Research Design and Methods: A
Cre-LoxP strategy was used to generate conditional knockout mice in which the Kif5b gene is specifically inactivated in pancreatic β-cells. Series of physiological and histological analyses were carried out in kif5b knockout mice as well as littermate controls. Results: Mice with β-cell specific deletion of Kif5b (Kif5bfl/−:RIP2-Cre) displayed significantly retarded growth as well as slight hyperglycemia in both non-fasting and 16 hr fasting conditions compared to control littermates. In addition, Kif5bfl/−:RIP2-Cre mice displayed significant glucose intolerance, which was not due to insulin resistance but related to an insulin secretory defect in response to glucose challenge. These defects of β-cell function in mutant mice were not coupled with observable changes in β-cell size, islet morphology or islet cell composition. However, compared to controls, pancreases of kif5bfl/−:RIP2-Cre mice exhibited both reduced islet size and increased islet number, concomitant with an increased total islet mass. Conclusions: In addition to being essential for maintaining glucose homeostasis and regulating β-cell function, Kif5b may be involved in β-cell development by regulating β-cell proliferation.

REFERENCES

1702/B81
Characteristics of Anti-asthmatic Agents from Chemical Libraries for Early Drug Discovery.
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Chronic asthma is characterized by inflammatory cell infiltration and tissue remodeling leading to subepithelial fibrosis. In this study, we investigated that LX519290 is one of candidate against inflammatory disease by examining various In Vitro and In Vivo assays. LX519290 demonstrated unique pharmacological profiles in antioxidant activity. In Vivo sensitization of C57BL/6 mice challenged with ovalbumin and immunohistochemistry of cytokine expression by lung inflammation were checked. Interestingly the compound has increased IFN-γ protein expression, whereas decreased IL-10 mRNA expression. Migration molecules such as ARF3 and N-WASP in PMA-induced mouse primary spleen cells were inhibited. Evidence from a mouse OVA model demonstrated that LX519290 inhibited the airway remodeling process, including immune cell inhibition, mucus by per-secretion and inflammatory cytokines. These results collectively suggest that LX519290 has potential in alleviating some asthmatic symptoms to mediate inflammatory events of the lung.

1703/B82
In Vitro Evaluation of Neuraminidase Inhibitors Using the Neuraminidase-Dependent Release Assay of Hemagglutinin-Pseudotyped Viruses.
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For the treatment of influenza virus infections, neuraminidase inhibitors (NAIs) that prevent the release of virus particles have been effective against most influenza strains. Several neuraminidase (NA) assays are available for the evaluation of NAIs. To understand the NA functions under physiological conditions, assays mimicking viral particle release should be useful. We have constructed retrovirus-based reporter viruses that are pseudotyped with hemagglutinin (HA) glycoprotein by transfection of producer cells using plasmids expressing retroviral gag-pol, influenza HA, NA, and firefly luciferase genes. Similarly to the life cycle of influenza viruses, the release of pseudotype viruses also requires neuraminidase functions. This requirement was used to develop an assay to evaluate NAI activities by measuring inhibition of pseudotype virus production at different NAI concentrations. The pseudotype virus release assay was used to determine the IC(50) values of Oseltamivir carboxylate, Zanamivir, and the novel phosphonate
congeners of Oseltamivir against N1 group neuraminidases and their H274Y Oseltamivir carboxylate-resistant mutants. The deduced IC(50) values obtained using the release assay correlated with those determined using the fluorogenic substrate 2′-(4-methylumbelliferyl)-alpha-d-N-acetylmuramic acid (MUNANA) and also correlated with the infectivity results.

**Host-Pathogen Interactions III (1704 – 1720)**

**1704/B83**

**FIV Resistance to Expression of a Tsg101 N-terminal Fragment Maps to the Env Glycoprotein.**

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Feline immunodeficiency virus (FIV) causes AIDS in domestic cats but is harmless to humans. Thus, FIV is an important model for the development of new vaccine and antiretroviral strategies, with additional applications in human gene therapy. Retroviral assembly and virion release pathways are driven by actions of the viral Gag protein and its interactions with host factors. At present, no clinical therapy that inhibits this critical phase of the viral life cycle has been developed. All Gag proteins bear short peptide motifs known as “late domains”, which bind elements of endosomal sorting complexes (ESCRT) to promote virus release. The PTAP motif in HIV-1 Gag binds an N-terminal domain in Tsg101, which is part of ESCRT-I. We have shown that FIV relies on a related PSAP motif in Gag to promote virus release in both human (HeLa) and feline (CrFK) cell lines. Overexpression of the Tsg101 N-terminal domain alone (TSG-5') specifically inhibits the release of HIV-1 and FIV. To target late domain function as a potential antiretroviral strategy, we engineered CrFK cells to express high levels of TSG-5', which severely impairs FIV replication. This is the first late-domain-specific inhibition of retroviral replication to be described. To explore possible mechanisms of resistance, we selected for FIV variants that replicate in CrFK/TSG-5' cells. Consistently, resistance-conferring mutations mapped to the FIV envelope (Env) glycoprotein; specifically, in variable loop 3 (V3) of the surface (SU) subunit and the distal heptad repeat (HR2) of the transmembrane (TM) subunit. These domains play critical roles in membrane fusion during entry of both FIV and HIV-1. Intriguingly, EM analysis of FIV-infected CrFK/TSG-5' cells shows long, thin extensions from the plasma membrane coated with mature virions. By immunofluorescence microscopy we also detected highly extended Gag-associated filaments, which may be related. Similar structures have been described that promote cell-cell transmission of HIV-1 and MLV along actin-based filopodia called “nanotubes” or “cytonemes”, which may depend upon Env-receptor interactions. We propose that FIV Env mutations allow escape from TSG-5' expression by enhancing cell-associated transmission.

**1705/B84**

**Association of Cytoskeletal and Hepatitis C Virus (HCV) Proteins in HCV Infection.**

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Background: Chronic hepatitis C (CHC) is a major cause of liver disease worldwide. A variety of host proteins interact with the HCV proteins. Using mass spectrometry we recently found that filamin a and vimentin are highly expressed in human hepatoma cell lines expressing HCV RNA and proteins compared to parental Huh7 cells. The definitive role of cytoskeletal proteins in HCV infection remains to be determined. In this study, our aim was to identify differentially regulated and expressed cytoskeletal proteins and their interaction with HCV proteins that might be used as potential biomarkers or targets of therapeutic interventions. Methods: We performed immunofluorescence (IF), Western blot, immunoprecipitation (IP) and quantitative RT-PCR of mRNAs to validate the proteomic profiling results and to delineate interactions between
cytoskeletal and HCV proteins in CNS3 [expressing HCV core to non structural (NS) 3 proteins],
9-13 (expressing NS3 to NS5B proteins), Con-1 cells (containing the full-length HCV genotype 1b
replicon) and in liver biopsies from HCV infected subjects vs. controls. Results: IF staining
revealed that HCV core, NS3 and NS5A-labeled speckles colocalized with filamin a but not with
vimentin. These results were confirmed in liver biopsies from HCV-infected subjects vs. controls.
IP showed interactions among filamin a and HCV core, NS3 and NS5A proteins. Con-1, CNS3
and 9-13 cells treated with Interferon alpha showed a dose-dependent decrease of cytoskeletal
and HCV proteins. NS3 and NS5A proteins were clustered only in perinuclear region when actin
filaments were depolymerized by cytochalasin B compared to disperse cytoplasmic and
perinuclear distribution in the no treatment group. Conclusions: Our findings that changes occur
in the cytoskeleton of hepatocytes in order to adapt to HCV protein expression following HCV
infection and suggest a novel and important role of cytoskeletal proteins in HCV infection and
progression. These results also demonstrate the utility of quantitative proteomics as a robust
discovery tool for the identification of differentially regulated proteins in HCV infected
hepatocytes. Acknowledgement: NIH grant RO 1 DK38825, contracts NO-1 DK92326 and UO-1
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1706/B85
The Host Cytoskeleton Exhibits Dramatic Alterations after Parvovirus Uptake.
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Parvoviruses are icosahedral non-enveloped single stranded DNA viruses with a genome size of
about 5 kb. Like with most DNA viruses, an efficient infection requires the virus to overcome
barriers within the cell in order to reach the nucleus and replicate its genome. Once viruses enter
the cell through mechanisms such as endocytosis, the next step is to travel a distance of often
100 capsid diameters to the nucleus. Although diffusion is efficient for movement over short
distances, it does not provide a means for directed long-distance transport. Therefore many
viruses use the host’s organized network of filaments to aid them in the mechanism of
intracellular trafficking, either for cytosolic transport of naked viral particles or for transport inside vesicles. During early infection with one of the smallest DNA viruses known, the parvovirus Minute Virus of Mice (MVM), we have found that the virus induces dramatic morphological changes in mouse fibroblast cells. This observed change in the shape of infected cells may be a result of the virus using the host’s organized network of three filaments, intermediate filaments
(IFs), microtubules (MTs), and microfilaments (MFs) to aid in the mechanism of intracellular
trafficking. Thus our present study focuses on MVM induced cytoskeletal alterations in relation to
the process of nuclear targeting during early MVM infection. Here we characterize the effects of
MVM on three cytoskeletal components, IFs, MTs, and MFs by immuno-fluorescence microscopy
of mouse fibroblast cells infected with MVM. Our results show that during early infection with
MVM, IFs are already cleaved, collapse, and accumulate on one side of the nucleus in close
proximity to the virus. MTs are also rearranged and recruited to the same side of the nucleus,
while MFs show no alteration. These alterations may be induced by the virus to increase the
efficiency of nuclear targeting, either by increasing the recruitment of MTs for viral transport to the
nucleus, or by specifically targeting the breakdown of IFs to prevent acidification of endosomes.
The use of such mechanisms by MVM shows the extent to which viruses may take advantage of
host mechanisms to increase the efficiency and spread of infection.

1707/B86
Role of Protein Kinase C Delta and Emerin in HSV-1 Nuclear Egress.
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The nuclear lamina is a meshwork of lamin proteins surrounding the nucleus and anchored to the
inner nuclear membrane (INM) via interactions with lamin associated proteins (LAPs). The lamina
is hypothesized to be a steric barrier to herpesvirus capsid access to the INM. Disruption of the
lamina, via mechanisms such as phosphorylation, is predicted to be essential in HSV-1 nuclear
egress. In HSV-1 infected cells, emerin, a LAP, is phosphorylated, redistributed, and hypermobile within the nuclear membrane. The viral kinase US3 and cellular kinase(s) sensitive to Rottlerin, a reported specific PRKCD (Protein Kinase C delta) inhibitor, are required for emerin phosphorylation and redistribution. TEM analysis of infected cells treated with Rottlerin resulted in increased accumulation of nuclear and no cell surface virions, suggesting that Rottlerin sensitive kinase(s) are required for nuclear egress of HSV-1. When factors involved in emerin modification, are missing or inhibited, viral growth is decreased to background titers. Knockdown of PRKCD protein levels did not have a significant effect on viral replication. Expression of a dominant negative (dn) PRKCD did not block emerin relocation in the same manner as Rottlerin treatment. PRKCDdn also had no significant effect on viral replication as compared to PRKCDwt. These results suggest that activity of Rottlerin sensitive kinase(s) is required for viral growth possibly due to a requirement for disruption of lamina components for primary envelopment. These data also suggest the absence of PRKCD activity does not induce a significant inhibition in HSV-1 replication possibly due to redundant functions of other kinases.

1708/B87
Nuclear Entry of Parvovirus Involves Cellular Mechanisms for Nuclear Envelope Breakdown.
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In order to promote efficient infection, viruses must target their genomes to specific compartments within the cell. In general, more is known about the intracellular trafficking of enveloped viruses as compared with non-enveloped viruses. Thus, we are using the parvovirus *Minute virus of mice* (MVM) as a model to study trafficking of non-enveloped viruses. Parvoviruses are small, single stranded DNA viruses that must enter the nuclei of their host cells to replicate successfully. However, the nuclear envelope (NE) acts as a barrier between the cytoplasm and the nucleus, and nuclear import is tightly regulated by the nuclear pore complexes (NPCs). Because of their small size, it has been assumed that parvoviruses enter the nucleus intact through the NPC. However, we have found that early during infection MVM causes transient disruption of the nuclear membranes. Using electron microscopy, we have also observed virus particles associated with the NE, and between the outer and inner nuclear membranes in close proximity to these disruptions. Our data indicate that MVM induces small disruptions of the nuclear membranes and enters the nucleus through the resulting breaks. We have performed microinjection and infection experiments in the presence of an array of pharmacological inhibitors in order to investigate the mechanism of MVM-induced NE disruption. We have found that the viral phospholipase A2 (PLA2) - the only known enzymatic domain on the parvovirus capsid - is not involved in causing NE disruption. Instead, the virus seems to use host cell enzymes such as caspases, proteases involved in causing nuclear envelope breakdown (NEBD) during apoptosis, to induce these nuclear membrane disruptions. However, complete apoptosis as detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) does not occur in infected cells until 48 hours after infection, once viral replication has occurred. We propose that parvoviral nuclear entry involves the hijacking of cellular factors to initiate a limited program for NEBD similar to that used by the cell during apoptosis. This is a novel type of mechanism that has never been described for the nuclear import of any virus or protein.

1709/B88
Immunomodulatory Activity of Alkyl Azelates as Assessed by Multiplexed Bead-Based Proteomic and Genomic Analyses of Inflammatory Biomarkers in a Human Skin Model.
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Objective: to evaluate the immunopharmacologic activity of mixed alkyl azelates (HF1107) and measure evoked biomarker modulation patterns in a human skin model using multiplexed...
proteomic and genomic tools. Methods: This study evaluated the immunomodulatory activity of HF1107 in human skin tissues challenged by a panel of chemical and biological irritants [croton oil, capsaicin, 1-chloro-2,4-dinitrobenzene, LPS, SDS and antigen preparations from M. ulcerans, P. acnes and methicillin resistant S. aureus] as compared to the activities of other counterirritants (indomethacin, dexamethasone and diphenhydramine). Levels of ATP, 45 cytokines, chemokines and PGE-2 in cultured media and tissue lysates were measured. Levels of RNA expression of 35 cytokines and chemokines were determined in fixed tissue. Tissues were also evaluated microscopically. Inter-group differences were assessed by Student’s t-test. Results: Multiplexed Immunoassays and QuantiGene Plex assays indentified patterns of both protein and RNA markers by the treatments based on highly significant (t < 0.001) intergroup differences. We observed significant modulation of biomarker expression levels in comparisons of tissues solely challenged with antigens to tissues that were antigen challenged and concurrently treated with HF1107. HF1107 was found to significantly modulate the expression of IL-1b, IL-6, IL-8, IL-10, IL-12p40, IL-17F and IL-23. Conclusions: Overexuberant inflammatory response of the host immune system to microbial infections is known to contribute to the morbidity and mortality in many human diseases. As some microbes exploit the host immune response to propagate and enhance infection, modulation of the host response may alter the natural course of microbial infections. HF1107 significantly modulates the production of a broad range of intra- and intercellular signaling molecules implicated in microbial diseases. These studies provided critical information on the pharmacological properties of the HF1107 and provided insights as to how it may be used to influence the pathophysiology of human diseases.

1710/B89 Placental Syncytiotrophoblast Resists Listeria monocytogenes Infection but Is Bypassed via Extravillous Cytotrophoblasts.

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Listeria monocytogenes is a well-studied pathogen that can cross the maternal-fetal barrier and is a useful model organism with which to probe the structure of that barrier. Past studies show that L. monocytogenes undergoes a significant bottleneck in trafficking from maternal organs to the placenta, and then again from placenta to fetus. Our work defines the barriers that cause these bottlenecks. L. monocytogenes can infect non-phagocytic cells by two means: direct invasion and cell-to-cell spread. The relative contribution of each to placental infection is controversial, as is the anatomical site of invasion. Here, we used quantitative microscopy to address these questions in first trimester placental explants that closely approximate the in Vivo structure of the human maternal-fetal interface, which is made of fetal cells (trophoblasts), maternal blood, and uterine tissues (decidua). Trophoblasts differentiate into subpopulations with distinct functions, e.g. syncytiotrophoblast, which is bathed in maternal blood and mediates gas and nutrient exchange, or extravillous cytotrophoblasts, which anchor the placenta in the decidua. Our analysis demonstrated that the syncytiotrophoblast was highly resistant to L. monocytogenes infection by either direct invasion or cell-to-cell spread from infected macrophages. Removal of the syncytiotrophoblast allowed L. monocytogenes access to newly vulnerable sites of invasion. Toxoplasma gondii was also unable to colonize the syncytiotrophoblast. Instead, extravillous cytotrophoblasts served as the portal of entry from both extracellular and intracellular compartments. After cytotrophoblast infection, subsequent bacterial dissemination to the fetus was hampered by further cellular and histological barriers. Extravillous cytotrophoblasts are located in the decidua and have little to no contact with maternal blood. The decidua is populated by high numbers of maternal leukocytes. Interestingly almost all pathogens capable of crossing the maternal-fetal barrier have intracellular life cycles. Thus, our observations suggest a novel
reason for this predisposition: transplacental transmission starts by colonization of the decidua, most likely via infected maternal leukocytes.

**1711/B90**  
**ABSTRACT WITHDRAWN**

**1712/B91**  
**Chlamydia trachomatis Infection Causes Mitotic Spindle Pole Defects Independently from Its Effects on Centrosome Amplification.**  
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*Chlamydiae* are gram negative, obligate intracellular bacterial organisms, and *Chlamydia trachomatis* is the etiologic agent of the most commonly reported sexually transmitted disease in the United States. Chlamydial infections have been epidemiologically linked to cervical cancer in patients previously infected by human papillomavirus (HPV). *Chlamydiae* undergo a biphasic life cycle that takes place inside a parasitophorous vacuole termed an inclusion. The inclusion associates very closely with host cell centrosomes, and this association is dependent upon the host motor protein dynein. We have previously reported that this interaction induces supernumerary centrosomes in infected cells, leading to multipolar mitotic spindles and inhibiting accurate chromosome segregation. Centrosome amplification and mitotic spindle defects are characteristic of many human cancers. High risk HPV type 16 oncoproteins E6 and E7 contribute to cervical cancer by cooperating to induce abnormal centrosome numbers; however, pre-cancerous cells subjugate aberrant spindle pole formation by clustering multiple centrosomes to form bipolar spindles. Our findings demonstrate that chlamydial infection causes mitotic spindle defects independently of its effect on centrosome amplification. Chlamydial infection dramatically increased the number of defective mitotic spindles in both the mouse neuroblastoma cell line N1E-115 and a human epithelial cell line transformed with HPV oncoproteins E6 and E7 (End1). Both these cell lines have intrinsic centrosome number defects. We show that chlamydial infection increases centrosome spread and inhibits the spindle assembly check point delay to disrupt centrosome clustering. These data suggest chlamydial infection exacerbates the consequences of centrosome amplification by inhibiting the cell’s ability to suppress the effects of these defects on mitotic spindle organization. We hypothesize that these combined effects on mitotic spindle architecture identifies a possible mechanism for *Chlamydia* as a cofactor in cervical cancer formation.

**1713/B92**  
**Identification of Adhesin-Like Receptors of Sporothrix Schenckii Involved in Adherence to Various Cell Types.**  
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Sporothrix schenckii is etiological agent of sporotrichosis, an emergent subcutaneous mycosis that can evolve to systemic complication in immunocompromised patient. The adherence of pathogens to host tissues is a initial step in the infection. We analyzing the biochemical and cellular basis of the fungal adhesion. Using different cell cultures lines (nervous, bone, lung, epithelium, and endothelial), we determine: 1) Adhesion kinetic of yeast to these cells. In 1 h of interaction of fungus with cells, S. schenckii present major adhesion capability to nervous, pulmonary and epithelial cells. 2) Using surface cell wall proteins labeling with sulfo-NHS-LC biotin and later extraction process with boiling in 2% SDS, this revealed about 14 different bands of surface proteins with molecular masses between 30 and 220 kDa, 3) Using ConA-HRP stain in blot, we determine the mannoproteins nature of this released molecules from cells walls. In other side, 4) to identify the surface glycoproteins of yeast cells of S. schenckii (SgSs) implicated in adhesion to different cells, we use ligand-like assays, to this, SgSs previous labeled with biotin,
were placed in interaction with the cells used in this study. The fungal biotinilated proteins that interacted with tissue, were identified in blot using streptavidin-hrp reaction. The result shown, SgSs involved in the recognition of the tissues are: a) Mr> 180, 115, 100, 90 in nervous cells, b) Mr> 115, 100, 90 in lung cells, c) Mr>220, 180, 115, 100, 90 epithelial cells, and d) Mr>115 kDa in endothelial and bone cells. Finally, 5) SgSs that participate in the sporothrical adhesion process, were used to produce polyclonal polyvalent antibodies. The antibodies had the capability to inhibit considerably the yeast cell adhesion to nervous, pulmonary and epithelial cells. We propose these surface molecules could be receptors like-adhesins and mediate in the adhesion to host cell.

1714/B93
Schistosomal Lipids Induce Activation of Human Eosinophils Through Different Signaling Pathways: The Role of PGD2 and Toll-Like Receptor 2.
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Introduction and Objectives: Remarkable accumulation of eosinophils is a characteristic feature of inflammation associated with allergic and parasitic disease, especially caused by helminthes. Infiltrating eosinophils are strongly implicated in the pathogenesis of these disorders by virtue of their capacity to release an array of tissue-damaging mediators. The link between infection and exacerbation of allergic diseases may consist of various pathways having multiple steps, and direct activation of eosinophils by microbe-derived molecules. In order to verify whether schistosomal lipids could activate innate immune response on human eosinophils we analyzed the expression of Toll-like receptors and lipid mediator production. Methods and Results: Lipids from adult worms of Schistosoma mansoni were isolated and purified through thin layer chromatography (TLC) and used to stimulate human eosinophils. Our results showed that stimulation of human eosinophils In Vitro by schistosomal lipids induced inflammatory mediator production, lipid body formation and an increase of TLR2 expression. Schistosomal lipids exhibited significant amounts of PGD2 detected by EIA and were able to induce a PGD2-dependent production of 14,15 LTC4 (eoxin) and LTC4 through CRTH2 receptors. An increase of PGD synthase expression in eosinophils upon stimulation was also observed suggesting that host-derived endogenous PGD2 may also participate in the reaction. By using neutralizing Abs to TLR2, we demonstrated that schistosomal lipids, mostly through lysophosphatidylcholine, induced TLR-2 dependent eosinophil lipid body formation and eoxin and LTC4 production. Conclusion: Taken together, our results showed that schistosomal lipids induce human eosinophil activation in vitro, indicating a role for these structures in enhancing the production of proinflammatory eoxins and inducing innate immunity through TLR2- and PGD2-dependent pathway in human eosinophils. Moreover, TLR2 may play an important modulatory role in host immune response to S. mansoni infection.

1715/B94
Modulation of Inflammatory Signaling by Azelaic Acid Esters in Human Subjects In Vitro and In Vivo.
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Objective: to evaluate effects of by azelaic acid esters on inflammatory signaling in human subjects using multiplexed proteomic and genomic techniques. Methods: We evaluated the ability of seven azelaic acid esters to counter chemical irritation induced by croton oil in a 3-dimensional human skin tissue model. Patterns of biomarkers modulated by the esters versus select dermatological drugs were assessed using multiplexed immunoassays of 45 cellular and
extracellular cytokines and chemokines, prostaglandin PGE-2, and ATP. Treated specimens were also formalin-fixed and paraffin-embedded; RNA levels of 35 cytokines and chemokines were determined. A topical application of an azelaic acid ester mix was evaluated in one human subject with seborrheic dermatitis. Effects of a single oral dose of the azelate mix on blood levels of 40 cytokines and chemokines were evaluated in two human subjects over 48 hours. Inter-group differences were assessed by Student’s t-test. Results: We observed a considerable variability in anti-inflammatory potency and differential patterns of biomarker modulation between the various azelates in the skin model. The anti-inflammatory potency of azelate esters generally paralleled increase of the alkyl chain length of the alcohol moieties. Based on unique “signature patterns” of biomarkers assessed by proteomic and genomic tools, we designed a proprietary mixture of azelate esters (HF1107) for suppression of the keratinocyte inflammatory response. When tested in human subjects, HF1107 markedly decreased redness in the perinasal area due to the seborrheic dermatitis; the effect persisted for over 60 days. HF1107 significantly yet transiently modulated blood levels of >50% of the measured analytes. Conclusions: This study demonstrated ability of azelaic acid esters to modulate local dermal inflammatory response in skin and provided a proof of concept for using mixtures of azelates for the treatment of dermal inflammation. In combination with antibacterial and antiviral activity of HF1107 (Streeper and Izbicka, unpublished results), the anti-inflammatory activity of the azelates warrants their further development for treatment of a broad range of human diseases.

1716/B95
Characterization of Pfexp-250, a Novel Plasmodium falciparum-Exported Protein.
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The pathogenesis of malaria, a prevalent and widely distributed infectious disease, is caused by the erythrocytic cycle of the parasite and is strongly correlated with export of Plasmodium falciparum proteins to the host cytosol and plasma membrane. P. falciparum causes the most severe form of malaria in humans, with several hundred million infections each year and 1-3 million deaths. While most intracellular parasites grow in an active host, the asexual stages of the malaria parasite develop in a cell devoid of organelles or of a protein trafficking system. P. falciparum must remodel the erythrocyte by exporting proteins into the host cytoplasm and membrane. We have identified a novel P. falciparum -exported protein, PFEXP-250, which lacks a canonical N-terminal signal sequence and the obvious motifs described for PEXEL/VTS-positive and negative proteins. Our data show that PFEXP-250 is expressed and is exported to the host erythrocyte at all stages of the intraerythrocytic cycle. Moreover, we have shown that PFEXP-250 colocalizes and interacts with glycophorin-binding protein 130 (GBP130), a soluble PEXEL-positive P. falciparum -exported protein, ring-infected erythrocyte surface antigen (RESA), P. falciparum skeleton-binding protein 1 (SBP1) and spectrin, a host cytoskeletal protein. This study identifies a novel P. falciparum -exported protein which interacts with PEXEL-positive and -negative P. falciparum proteins and the host cytoskeleton. Because PFEXP-250 lacks the motifs described in PEXEL/VTS-positive and -negative proteins, these data raise the likelihood of an additional export pathway in P. falciparum.

1717/B96
Development of a Highly Potent Rabies DNA Vaccine: Towards a Radical Change.
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Rabies is progressive fatal encephalitis. WHO estimates 55,000 rabies deaths and 10 million post-exposure prophylaxis world wide every year. High cost of current cell culture derived vaccines is driving the quest for newer vaccine formulations; DNA vaccines being most promising amongst them. Methods: We aimed to explore strategies of antigen trafficking of DNA vaccines to various cellular compartments aiming at improving both humoral and cellular immunity. These strategies include use of signal sequences Tissue Plasminogen Activator, Lysosomal Associated
Membrane Protein-1 and their combination for enhancing the CD4+ T cell and antibody response; and Ubiquitin for enhancing CD8+ response. This was followed by investigation of immune enhancement strategies. Results: The potency of modified DNA vaccines assessed by total antibody response, antibody isotypes, cytokine profile, rabies virus neutralizing antibody (RVNA) titer and protection conferred against In Vivo challenge; was enhanced in comparison to native unmodified vaccine. LAMP-1 tagged DNA vaccine (pgp.LAMP-1) showed promising response in eliciting rabies virus neutralizing antibody response. Its route of administration and dosage schedule were optimized. Further, it was supplemented with adjuvants to enhance its potency. The optimized DNA vaccine formulation elicited robust neutralizing antibody titer (32 IU/ml) and conferred complete protection against challenge with Challenge Virus Standard (CVS) strain of rabies virus. Additionally, post-exposure therapy was evaluated. Rabies was experimentally induced; after which, therapy was initiated. Five doses of the optimized DNA vaccine formulation conferred complete protection from rabies in BALB/c in contrast to 80% protection with five doses of the conventional Rabipur rabies vaccine. Conclusion: This vaccine formulation is highly potent and confers complete protection on pre- and post-exposure in BALB/c mice. Acknowledgments: We acknowledge Department of Biotechnology, Government of India for providing financial assistance for this work.

1718/B97
Ras Activation Contributes to Microbe-induced Cholangiocyte Inflammatory Responses through Transcriptional Repression of the microRNA let-7i.
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Cholangiocytes, the epithelial cells lining the bile ducts, are periodically exposed to potentially injurious microbes. As a result, cholangiocytes actively participate in microbe-associated proinflammatory responses in the liver, yet the molecular mechanisms regulating these processes remain largely unknown. We previously showed that infection of cultured human cholangiocytes with the protozoon parasite, Cryptosporidium parvum, represses let-7i, a microRNA that targets Toll-like receptor-4, in an NFκB-dependent manner. Here, we address the role of the small GTPase Ras, a molecular target of the let-7 family, in microbe-induced suppression of let-7i and the promotion of pathogen-induced inflammatory responses of cholangiocytes. We demonstrate, using cultured human cholangiocytes and a GST-Ras Binding Domain activation assay, that TLR agonists against plasma membrane TLRs (TLR 1, 2, 4, and 5) robustly activate N-Ras, but not other p21Ras isoforms, while endosomal TLR agonists do not. Indeed, the specific TLR4 agonist, LPS, rapidly (<10 min.), and persistently (>24 hr.) induces N-Ras activation, and promotes phosphorylation of the downstream target, ERK1/2. We further demonstrate, using a dual luciferase based promoter assay, that microbial stimuli suppress expression of the microRNA, let-7i (~2-fold; p<0.05), while an N-Ras si-RNA blocks this suppression. Furthermore, microbe stimulated cholangiocytes exhibit increased N-Ras expression, yet, the microbe-induced expression of N-Ras, and corresponding increase in ERK1/2 phosphorylation, is blocked when infections are performed following transfections with a let-7i precursor molecule. Finally, C. parvum infection or treatment with LPS induces both increased cholangiocyte chemokine/cytokine production and proliferation. Transfection with an N-Ras siRNA diminishes microbe-induced cytokine production (e.g. IL-8 reduced over 50%) and proliferation (~ 2-fold; p<0.05). Together, our results suggest that let-7i expression is regulated by and regulates TLR-dependent N-Ras activation, which contributes to the cholangiocyte microbe-induced proinflammatory response.

1719/B98
Trans-sialidase Activity Mediates G-protein Dependent Cell Entry.
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Trans-sialidase activity catalyses the transfer of alpha-(2-->3)-sialic acids from donor to acceptor glycoconjugates. It is a unique activity specific to the surface of the invasive form of the parasite Trypanosoma cruzi. Trans-sialidases and trans-sialidase like proteins form an abundant and heterogeneous family of proteins expressed on the parasite surface that have been implicated in host parasite interaction but only some of which are enzymatically active. In these experiments we have coated active (TcTS) and inactive (TcTS2V0) recombinant trans-sialidase onto latex beads and followed their uptake by MDCK II cells. Both proteins induce cholesterol-dependent actin-mediated entry of beads. Laurdan microscopy showed relatively higher liquid order at the plasma membrane interface with the coated beads and fluorescence imaging showed an accumulation of caveolin-1 at both the bead interface and the subsequent bead vacuole. TcTS coated beads showed 3 fold higher levels of attachment and entry than the TcTS2V0 coated beads. This increased entry was ablated by pertussis toxin, thus identifying parasite trans-sialidase activity as a modulator of the host cellular response via G protein signalling. Thus, our results demonstrate that trans-sialidase mediated uptake is not solely activity dependent and that active and inactive protein may well mediate cross-linking of host glycoconjugates driving raft formation and caveolin mediated (macro)endocytosis of the beads; however, trans-sialidase activity markedly increases cell entry via increased adhesion and the induction of additional complementary cellular responses downstream of G protein signalling such as exocytosis.

1720/B99
Cryptococcal Cell Morphology Affects Host Cell Interactions and Pathogenicity.
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Cryptococcus neoformans is a common life-threatening opportunistic human fungal pathogen. C. neoformans grows as a budding yeast In Vitro with typical cell sizes ranging from 5-10 μm in diameter. Early reports suggested the presence of enlarged cells in human infections yet the identity of these cells and their role in virulence remained uncharacterized. Changes in cellular morphology are also observed in the mouse inhalation model of cryptococcosis. The goal of our studies was to identify In Vivo morphological changes and their affect on pathogenesis. In the mouse model, cell size increases were observed in the lungs, producing cells up to 100 μm in diameter. These enlarged “titan” cells accounted for 20% of the cells in the lungs. Titan cell formation could be stimulated by coinfection with strains of opposite mating type, and ste3aΔ pheromone receptor mutant strains had reduced cell enlargement. Titan cell formation was found to be regulated by G-protein coupled receptor (GPCR) signal transduction pathways associated with pheromone sensing and stress response. Analysis of titan cells revealed uninucleate, polyploid cells that reproduced by budding, suggesting alterations in the regulation of cell growth and mitosis. Titan cell formation also affected host-pathogen interactions by reducing phagocytosis by host mononuclear cells and was correlated with reduced dissemination within the host. These results describe a novel mechanism by which C. neoformans undergoes morphogenesis to evade host phagocytosis, leading to the hypothesis that titan cell formation allows survival of a subset of the population and plays a unique and specialized role during infection.

Cell Biology of the Immune System II (1721 – 1737)

1721/B100
Identification of a Motif in TLR8 Ectodomain Required for Ligand Recognition.
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Toll-like receptors (TLRs) are key sensors in innate immune cells for detection of diverse pathogens. TLRs are type I transmembrane receptors containing an ectodomain, a
transmembrane region, and a cytoplasmic TIR domain. The TLR ectodomain contains multiple copies of leucine-rich repeats (LRRs) that are arranged to form a horseshoe-shaped solenoid structure with the α-helices on its outer convex surface, and the parallel β-sheets on its concave surface for ligand interaction. The TIR domain provides a key site for interaction with TIR domain-containing adapter proteins to initiate downstream signaling pathways. TLR8 belongs to a subfamily comprising TLR7, TLR8 and TLR9. This TLR mediates anti-viral immunity by recognizing ssRNA viruses, and triggers potent anti-viral and antitumor immune responses upon ligation by synthetic small molecular weight ligands. Generally, therapeutic agents are tested preclinically in rodent animal models and the resulting data are then transferred to other species for further investigation. Interestingly, while activation of human (h)TLR8 elicited potent immune responses, mouse (m)TLR8 was first reported as a nonfunctional receptor and subsequently shown to be functional upon stimulation with small molecular agonists in the presence of polyT-ODN. The molecular basis for this distinct recognition is unclear. In the present study, we established cell based TLR8 activation assay to investigate the activation of hTLR8 and mTLR8. The results showed that compared to hTLR8, mTLR8 was not responding to ligand stimulation, and exhibited a lower activity to ligand stimulation in the presence of polyT-ODN, even though the cytoplasmic domain of mouse TLR8 has a comparable activity as that of human TLR8 in initiation of downstream signaling. Further investigation with deletion mutation and point mutation analyses suggested that a motif in ectodomain is essential for this distinct ligand recognition in hTLR8, whereas this motif is not required for intracellular localization and self-dimerization of this receptor.

1722/B101
Activation of GPR30, a Novel Estrogen Receptor, Results In Acute Decreases in TLR4 Expression on Murine Macrophages.
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To date, the rapid signaling events and the genomic actions of estrogen have been attributed to the engagement of the classical nuclear hormone estrogen receptors, ERα and ERβ. However, the recent discovery of GPR30, a G protein-linked membrane estrogen receptor that also mediates rapid non-genomic events, may underlie alternative estrogen effects. GPR30 has been found to mediate numerous rapid cellular events in target cells following receptor ligation, such as calcium mobilization and MAPK activation. In this study we have investigated the role of GPR30 on macrophage expression of Toll-like receptor 4 (TLR4), a key pattern recognition receptor for detection of bacterial lipopolysaccharide. We show that both primary murine peritoneal macrophages and RAW 264.7 cells, a macrophage-like cell line, express GPR30 message and protein. Furthermore, we report that rapid treatment of RAW 264.7 cells with estrogen results in a significant decrease in cell surface TLR4 expression within one hour. However, cell surface TLR4 expression returns to baseline levels after 12 hours. Importantly, rapid treatment of macrophages with ICI 182780, an ERα and ERβ antagonist that functions as a GPR30 agonist, and G1, a GPR30 specific agonist, result in similar decreases in TLR4 expression within one hour. While these data appear to contradict our recent studies showing that chronic in vivo exposure to estrogen augments expression of TLR4 on peritoneal macrophages, we suggest that estrogen acts via multiple receptor types to induce kinetically dissimilar responses that can influence infectious disease susceptibility. As such, estrogens may be able to reduce the rapid and acute inflammatory events associated with an overactive host response, such as that which occurs during sepsis, without compromising long-term defense against infectious organisms.

1723/B102
Evil Twins: The Balance between T Cell Activation and Anergy Is Established by Two Cytohesin Family Proteins with Opposing Functions.
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The activation of T cells in response to foreign antigen induces cytokine production (e.g. interleukin 2), antigen specific T cell clonal expansion and differentiation. The complex mechanism of T cell activation is tightly controlled by a variety of intracellular signaling pathways which are still not fully understood. We show here that the cytoplasmic GEF protein cytohesin1 regulates important intracellular events in the course of T cell activation. Employment of RNAi and reporter gene assays reveals that cytohesin1 expression positively regulates TCR induced transcriptional activation of the IL2 gene via activation of the AP1 transcription factor complex. Cytohesin1 mediates positive regulation of AP1 by selectively activating the p38/JNK, but not the ERK1/ERK2 branch of the MAP kinase signaling cascade. The ability of cytohesin1 to activate p38/JNK signaling depends on the GEF function of the protein, since overexpression of the dominant negative GEF mutant E157K, or treatment with cytohesin specific chemical GEF inhibitors completely blocks TCR induced activation of p38. Interestingly, overexpression of the homologous protein cytohesin3, which lacks a carboxy-terminal serine phosphorylation site present in cytohesin1, represses IL2 promoter activation, suggesting that this highly related protein is a direct antagonist of its "brother" cytohesin1. Consistently, the inhibition of T effector cell proliferation by CD25+ FoxP3+ regulatory T cells is specifically abrogated in cytohesin3 knock-down effector cells. Fully in line with all the findings described above, we demonstrate that cytohesin3 expression is strongly upregulated in various types of anergic T cells. We propose here that cytohesin3 acts as important factor for anergy/tolerance induction or maintenance through functional inhibition of the cytohesin1/p38 MAP kinase/AP1 signaling axis.

1724/B103
PKCθ Has Selective Roles for TH17 Cell Function In Vivo and In Vitro.
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PKCθ is involved in the signaling pathway for IL-2 production in T cells and plays important roles in T cell activation, cytokine production, and proliferation. PKCθ regulates pulmonary inflammation and airway hyperresponsiveness in addition to disease models of experimental autoimmune encephalomyelitis (EAE), experimental autoimmune myocarditis (EAM), arthritis, and colitis. Using mice deficient in PKCθ and a selective PKCθ inhibitor, we asked whether PKCθ plays a role in Th17 cell differentiation or function. In a short term In Vivo anti-CD3 administration model, in which serum cytokine titers are assayed three hours post-stimulation, PKCθ knock-out (KO) mice had a trend toward elevated titers of IL-17 and IL-22, but reduced IL-2 and TNFα, compared to wild type (WT) mice. Thus, IL-17 - producing cells were present in PKCθ KO mice, and could be activated upon stimulation with anti-CD3. Because IL-2 antagonizes Th17 cell differentiation, the trend toward higher IL-17 titers in anti-CD3-treated PKCθ KO mice may be secondary to their relative deficiency in IL-2. Consistent with this, treatment of WT mice with a selective PKCθ inhibitor reduced serum titers of IL-2 and TNFα 3 hours post anti-CD3 administration, but did not affect titers of IL-17. To examine effects of PKCθ on In Vitro Th17 differentiation, CD4+ T cells were isolated from naive WT or PKCθ KO mice, and stimulated for 7 days with a cocktail of cytokines. The yield of Th17 cells was comparable when differentiated from CD4+ T cells of PKCθ KO or WT mice. Upon TCR restimulation of the differentiated Th17 cells, addition of PKCθ inhibitor did not affect production of IL-17, but severely reduced production of IL-2 and TNFα from WT but not the PKCθ KO cells. Taken together, our findings demonstrate that PKCθ is not directly involved in Th17 differentiation or IL-17 production, but is involved in IL-2 and TNFα production.

1725/B104
APLP2 Expression Regulates Endocytosis of MHC-Peptide Complexes through a Clathrin-Dependent Pathway.
Immune responses against viruses and tumors rely on the presentation of viral and tumor-derived peptides to cytotoxic T-lymphocytes by cell surface receptors known as major histocompatibility complex (MHC) class I molecules. Little is known about the proteins regulating trafficking of MHC-peptide complexes to and from the cell surface. One candidate protein that we have shown to be associated preferentially with peptide-bound forms of the murine MHC class I molecule Kd is amyloid precursor-like protein 2 (APLP2). In our recent study, we have demonstrated that overexpression of APLP2 enhances endocytosis of Kd, leading to its reduced surface expression and stability. Our findings indicate that APLP2 associates with Kd both at the cell surface and in endocytic vesicles. To elucidate the mechanisms by which increased APLP2 expression causes enhanced endocytosis of Kd molecules, we analyzed how APLP2 itself undergoes endocytosis. Our findings indicate that APLP2 is internalized in a clathrin-dependent manner, as a dominant negative dynamin II mutant and the C-terminal tail of AP180 (both inhibitors of clathrin-dependent endocytosis) block its internalization. However, endocytosis of Kd is not blocked by these inhibitors, consistent with findings by others that MHC class I molecules are internalized mainly through a clathrin-independent pathway. Furthermore, the APLP2 cytoplasmic tail contains consecutive tyrosine-based NPXY and YXXØ motifs that can potentially bind to adaptor protein AP-2. Knock-down of the AP-2 µ2 subunit by siRNA or mutation of the tyrosine in the NPXYXXØ sequence leads to severely impaired internalization of APLP2, indicating the functionality of this motif in APLP2 endocytosis. Interestingly, this APLP2 Y755A mutant, unlike wild-type APLP2, fails to enhance the endocytosis of Kd molecules. Moreover, expression of dynamin II K44A also blocks the enhanced endocytosis of Kd induced by APLP2 overexpression. These findings suggest a model whereby increased expression of APLP2 causes recruitment of clathrin machinery at the site of APLP2 association with Kd molecules at the cell surface, resulting in enhanced uptake of Kd in a clathrin-dependent manner.

**1726/B105**

Upregulated IL-19 May Induce Hyporesponsiveness of CD4+ T Cells in Patients of Cardiac Surgery with Cardiopulmonary Bypass.

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Patients undergoing cardiac surgery with cardiopulmonary bypass (CPB) may encounter immunosuppression and infectious complications. Interleukin (IL)-19 was markedly induced after CPB. The present study demonstrates the effect of IL-19 on suppression of CD4+ T cell functions of patients undergoing cardiac surgery with CPB. Blood samples were withdrawn from 22 patients undergoing elective cardiac surgery before anesthesia (pre-CPB) and at 24 h post-CPB. Serum cytokine levels were analyzed using ELISA. CD4+ T cells were isolated, cultured and assayed for proliferation, cytokine production and Foxp3 mRNA expression with or without IL-19 treatment. Serum levels of IL-10 and IL-19 were significantly increased and correlated after CPB. The levels of IL-19 receptors mRNA in CD4+ T cells were upregulated after CPB. With PMA/ionomycin stimulation, the percentage of cell proliferation and the levels of IFN-γ production were lower in CD4+ T cells isolated from patients post-CPB than those from patients pre-CPB. In addition, post-CPB CD4+ T cells inhibited pre-CPB autologous CD4+ T cell proliferation. All these effects were enhanced in high-IL-19-production patients. Administration of recombinant human (rh)IL-19 led to reduced cell proliferation and IFN-γ production in CD4+ T cells isolated from healthy volunteers. Foxp3 mRNA expression in CD4+ T cells was significantly increased after CPB. Foxp3 mRNA was induced in IL-19-treated CD4+ T cells which also reduced proliferation of autologous non-treated CD4+ T cells. The present study demonstrates that IL-19 may reduce T cell responses
and induce the regulatory function of CD4+T cells. Induced IL-19 production in patients of cardiac surgery with CPB may contribute to hyporesponsiveness of cell-mediated immunity.

1727/B106
Immunomodulation Allows Beta-pancreatic Islets Regeneration after Multipotent Mesenchymal Stromal Cells Endovenous Administration into Type 1 Diabetic Mice.
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Type 1 diabetes affects 17 million of people worldwide. Nowadays, any treatment allows accurate blood glucose levels control. Thus, patients develop severe diabetic complications. Recently, we demonstrated that the administration of 0.5x10^6 mesenchymal stromal cells (MSC) into mice with type 1 diabetes (DMT1) reverted hyperglycemia, increased insulinemia, and reduced glycated-hemoglobin. This reversion correlated with the recovery of beta-pancreatic islets number, morphology and function. The aim of the present work was to evaluate the contribution of MSC immunomodulatory potential, to beta-pancreatic regeneration observed in DMT1 mice. First, we assessed -by qRT-PCR- the expression of molecules that enhanced [IL1b, TNF, INFg, TGFb1, MCP1, ICAM1] or prevent [IL2, IL4, IL5, IL10] the destruction of beta-pancreatic islets. In the pancreas of untreated DMT1 mice the expression of pro-inflammatory molecules was higher than in normal animals. Together, the expression of pancreas-protective genes was diminished. However, 7 and 65 days post-administration, IL1b, MCP1, ICAM1, IL2 and IL5 were expressed at basal levels and IL4 and IL5 were highly expressed in the pancreas of MSC treated DMT1 mice. Second, we analyzed -by flow cytometry-, the presence of protective regulatory T cells (Treg) in blood, spleen and bone marrow of DMT1 mice treated or not with MSC. at days 7 and 65, a significant increase in Treg was observed in spleen and bone marrow of MSC-treated mice compared to untreated DMT1 or normal mice. Third, we determined -by flow cytometry- the distribution of donor MSC in DMT1 receptors. for this we administered MSC that express GFP (MSCGFP) and tracked in different organs. Seven days post-administration, MSCGFP were mainly found in Peyer patches, in inguinal lymph nodes and bone marrow. Sixty-five days post-administration, MSCGFP were present almost exclusively in Peyer patches. Our results show that in DMT1 mice, donor MSC home into secondary lymphoid organs, induce Treg generation, reduce the activity of autoreactive T cells and maintain high level of pancreatic protective factors. Hence, the immunomodulation promoted by the administration of MSC allows the regeneration of beta-pancreatic islets.

1728/B107
Porcine Endothelial Cell Membrane Antigens Recognized by Natural Human Antibodies.
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The interaction of natural human antibodies with porcine cell surface antigens is one of the key barriers against utilizing pig organs for xenotransplantation. Endothelial cells lining the pig liver vasculature represent the first point of contact for the human immune system. Natural antibodies in human circulation recognize antigens expressed on porcine liver sinusoidal endothelial cells (LSEC) resulting in rejection of the pig liver. Many of these antibodies are directed towards the terminal carbohydrate gal-alpha-1,3-gal (gal) that is ubiquitously expressed in the pig. Not surprisingly, the creation of a gal knock out (KO) pig significantly improved the survival of the porcine xenograft but it failed to completely eliminate natural antibody mediated rejection. Our goal is to identity those antigens other than gal recognized by the natural human antibodies so that these can be modified or eliminated from the pig genome. LSEC were isolated from the liver of a wild type Yucatan mini-pig by collagenase perfusion. LSEC membrane proteins were resolved using proteomic techniques and then exposed to human serum whose natural antibody profile had been characterized quantitatively and qualitatively by ELISA. Porcine membrane
proteins recognized by natural human IgM and IgG were visualized by near-infrared imaging. Using MALDI TOF TOF mass spectrometry we have identified at least seven previously unknown porcine liver membrane antigens recognized by natural human antibodies. We compared our identified proteins to gal positive proteins by lectin blot analysis and total glycoprotein analysis. While, many proteins are galactosylated, a significant number of proteins that were positive on human serum blots are not galactosylated or glycosylated. This suggests that natural antibodies directed against protein epitopes as well as gal and other carbohydrates mediate organ rejection. To conclude, we have developed a technically superior strategy for antigen identification relevant to xenotransplantation. We are currently working on repeating this strategy on samples from gal KO pigs.

1729/B108
Transcriptomic and Proteomic Analyses Reveal the Effect of Echinacea purpurea Extract on Trafficking Activity in Mouse Dendritic Cells.
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In recent years, a variety of Echinacea species have been used as nutraceuticals or candidate botanical drugs for “immunostimulation”, but scientific evidence for their role in healthcare is still controversial. In this study, we used primary cultures to analyze the possible genomic and proteomic effects of an Echinacea purpurea phyto-extract on mouse bone marrow derived dendritic cells (BMDCs), as a follow up and hopefully a modeling system of our previous study using human DCs. Phytocompound mixture extracted from the butanol fraction (BF) of the stem and leaf (S+L) tissues of E. purpurea, termed [BF/S+L/Ep], containing stringently defined bioactive phytocompounds was used here systematically as previously report. Treatment of BMDCs with [BF/S+L/Ep] did not significantly influence the phenotypic maturation activity of dendritic cells (DCs). Affymatrix DNA microarray and bioinformatics analyses of genes expressed in [BF/S+L/Ep]-treated DCs after 4 or 12 h treatment revealed that the majority of the responsive genes were related to cell adhesion or motility (Cdh10, Itga6, Cdh1, Gja1 and Mmp8), chemokines (Cxcl2, Cxcl7), and signaling molecules (Nrxn1, Pkce and Acss1). By using TRANSPATH database analyses on genes expressed in DCs, we predict the JNK, PP2C-α, AKT, ERK1/2 or MAPKAPK pathways as the putative targets of [BF/S+L/Ep]. Proteomic analysis showed that expression of certain metabolism, cytoskeleton, and NF-kB signaling-related proteins in test BMDCs were differentially regulated by treatment with [BF/S+L/Ep]. An In Vivo DCs trafficking assay then showed that [BF/S+L/Ep]-treated DCs, as shown for lipopolysarchride-treated DCs, can migrate more effectively to the peripheral lymph node and spleen tissues than do the untreated DCs. These results suggest that [BF/S+L/Ep] could drastically affect the mouse DCs mobility in immune systems. The bio-informatics deduced signaling networks and molecular switch targets provided in this study maybe employed as potential targets for future clinical or nutraceutical applications on use of medical herbs.

1730/B109
Taurine Chloramine Produced by Neutrophil Protects Macrophages from H2O2-derived Cytotoxicity by Induction of Antioxidant Enzymes like HO-1, Prx-1, Trx-1 and Catalase.
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Taurine chloramine (TauCl) is produced in the activated neutrophils by reaction between the stored taurine and the newly overproduced hypochlorous acid, the product of neutrophilic myeloperoxidase halide system intended to kill the phagocytosed bacteria. TauCl has been shown to exert both anti-inflammatory and cytoprotective effects in inflammatory tissues by inhibiting the production of inflammatory mediators such as NO, O2-, ONOO-, and H2O2 by the activated phagocytes. Recently, we reported that TauCl induced heme oxygenase 1 (HO-1) expression and elevated HO activity in RAW 264.7 macrophages. HO degrades the potentially
damaging free heme to biliverdin, iron and carbon monoxide to inhibit Fenton reaction and also to provide the anti-inflammatory and antioxidant actions. In this study, we investigated whether TauCl could protect cells against the hydrogen peroxide (H2O2)-derived cytotoxic effect in RAW 264.7 macrophages. Macrophages exposed to exogenous H2O2 are known to undergo apoptosis and death. However, macrophages pretreated with TauCl were protected from the H2O2-derived apoptosis and death and increased cell viability in a time- and concentration-dependent manner of TauCl pretreatment. TauCl lowered cellular glutathione (GSH) level initially, which increased the expression of Nrf2-regulated cytoprotective anti-oxidant enzymes like peroxiredoxin (Prx)-1, thioredoxin (Trx)-1, HO-1. TauCl also increased catalase expression. Furthermore, TauCl elevated cellular GSH level 4 h after the initial depletion and maintained the increased GSH level for up to 12 h. We also observed that TauCl inhibited the H2O2-derived activation of caspase-3 in macrophages and protected the cells from apoptotic death. These results indicated that TauCl produced by the activated neutrophils at the site of inflammation could protect the surrounding tissues and macrophages from cytotoxicity caused by oxidants like H2O2 and ONOO- by inducing the expression of several cytoprotective antioxidant proteins like HO-1, Prx-1, Trx-1 and catalase in macrophages.

**1731/B110**

*Shedding of FcγRIIib Reduces Human Neutrophil Bactericidal Activity.*

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Fcγ receptor type IIb (FcγRIIib, CD16b) is a GPI-anchored protein that binds the Fc domain of IgG and is highly expressed on human neutrophils. Neutrophils activated by chemoattractants, such as fMLP, shed FcγRIIib resulting in its release into plasma and other body fluids. We have developed a sensitive ELISA assay that enables us to measure soluble FcγRIIib in normal human plasma and found concentrations ranging from 100 ng/ml to 200 ng/ml. We have used this assay to examine the effect of chemoattractants, enzymes, and activators of neutrophil function on FcγRIIib shedding. We report that 10⁶ human neutrophils incubated for 90 min at 37°C with 10⁻⁷ M PMA, 10⁻⁷M fMLP, C5a (produced by incubation of IgG-opsonized *S. epidermidis* in human plasma), or 6.25 mUnits PI phospholipase C (an enzyme that specifically cleaves GPI-linkages), in 1 ml phosphate buffered saline containing 0.1% BSA and 5.5 mM glucose released about 5 ng of FcγRIIib/10⁶ neutrophils. In contrast, addition of 10⁻⁷M LTB4 did not stimulate any FcγRIIib release above background, indicating that not all chemoattractants promote FcγRIIib release. Neutrophils pre-treated with 6.25 mUnits of PLC and incubated for 90 min at 37°C in stirred suspensions with 10⁵ cfu/ml human serum-opsonized *S. epidermidis* killed only 50% of these bacteria, compared to 98% killing by untreated neutrophils under the same conditions. Thus, chemoattractant-mediated release of FcγRIIib from neutrophils may alter the cells’ bactericidal activity.

**1732/B111**

*Surface Acoustic Waves Enhance Human Neutrophil Chemotaxis through Fibrin Gels and Neutrophil Killing of *Staphylococcus epidermidis* in Biofilms and Fibrin Gels.*

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Bacterial biofilms are the leading cause of antibiotic-resistant infections associated with implanted medical prostheses. Consequently, surgical removal of the infected prosthetic device is often required to clear the infection. This can be debilitating and costly. Repeated application of surface acoustic waves (SAW) to urinary catheters in rabbits reduced biofilm formation and helped resolve biofilm infections (Hazan, Z., et al., Antimicrob Agents Chemother, 50:4144-52, 2006). However, the mechanisms responsible for these beneficial effects are unknown. We have examined the effects of SAW (0.3 mW/cm²) on human neutrophil (PMN) chemotaxis through, and

SAW significantly (p <0.05) enhanced chemotaxis through fibrin gels in response to C5a and LTB4, and killing of 10^5-10^6 cfu human serum-opsonized *S. epidermidis* ml fibrin gel by 4 x 10^6 normal human PMN/ml fibrin gel. SAW also significantly enhanced (p <0.05) PMN killing of serum-opsonized *S. epidermidis* in biofilms formed on silicone wafers *in vitro*.

**1733/B112**

*Anthrax Lethal and Edema Toxins Do Not Directly Affect Human Platelet Function.*

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Blood vessel leakage and hemorrhage are prominent clinical manifestations of systemic anthrax. Platelets are anuclear cells that aid in hemostasis and a defective platelet function has been proposed as an explanation for mediastinal and pleural hemorrhage in anthrax. We have examined the direct effects of *Bacillus anthracis*’ lethal and edema toxins on human platelets. Western Blot analysis reveals that anthrax lethal toxin (LT) fails to cleave its anticipated target MEK1 and fails to block phosphorylation of the downstream substrate Hsp27 in human platelets. Anthrax edema toxin (ET) fails to stimulate intracellular cyclic adenosine monophosphate (cAMP) levels in human platelets. Furthermore, flow cytometry analysis reveals that neither LT nor ET inhibit P-selectin surface expression. The absence of these effects is explained by the finding that protective antigen (PA) fails to bind to or be internalized by human platelets. This lack in binding is due to an absence in the two PA receptors: tumor endothelial marker 8 (TEM8) and capillary morphogenesis gene 2 (CMG2) on human platelets. We conclude the hemorrhagic clinical manifestations of systemic anthrax cannot be attributed to the direct actions of anthrax ET or LT on human platelets.

**1734/B113**

*Macrophages Create a Lysosomal Synapse to Digest Aggregated Lipoproteins.*

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A critical event in atherogenesis is the interaction of macrophages with subendothelial lipoproteins. Although most studies model this interaction by incubating macrophages with monomeric lipoproteins, macrophages In Vivo encounter lipoproteins that are aggregated and avidly bound to the extracellular matrix. The physical features of the lipoproteins require distinctive mechanisms for their uptake. In particular, unlike monomeric LDL the uptake of aggregated low density lipoprotein (agLDL) does not involve receptor-mediated endocytosis but rather the aggregate is sequestered in deep invaginations at the cell surface, termed surface connected compartments (SCCs). We demonstrate that the SCC functions as a lysosomal synapse. Data will be presented that show delivery of lysosomal contents to SCCs via targeted exocytosis. The exocytosis depends on both intracellular calcium levels and microtubules as treatment with BAPTA-AM or nocodazole inhibits the delivery of lysosomal contents to SCCs. SCCs are LAMP-1 positive indicating that in addition to functioning as a source of hydrolases, lysosome exocytosis also serves as a supplemental source of membrane for compartment formation. We used time-lapse confocal fluorescence imaging to demonstrate the acidification of SCCs by vacuolar ATPases, thereby enabling functioning of lysosomal hydrolases. We observe transient sealing of portions of the compartments allowing formation of an ‘extracellular’ proton gradient. Further, an increase in free cholesterol is observed in aggregates contained in SCCs. Thus, cholesteryl ester hydrolysis can occur extracellularly in a specialized compartment, a lysosomal synapse, during the interaction of macrophages with agLDL. This novel endocytic pathway may more accurately reflect events that occur when macrophages encounter subendothelial lipoproteins in vivo. Further, macrophage formation of an extracellular lytic compartment parallels other biological systems, such as osteoclast bone resorption, and thus the lysosomal synapse may represent a generalized response of monocyte derived cells.
1735/B114

S100B/RAGE-dependent Chemoattraction of Microglia.

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The Ca2+-binding protein of the EF-hand type, S100B, is abundantly expressed in astrocytes where it has been implicated in the regulation of cell shape, migration, differentiation and proliferation (Biochim Biophys Acta 1793 (2009) 1008-1022). Besides having intracellular regulatory activities, S100B also exerts extracellular effects. Indeed, astrocytes release the protein constitutively and, to a larger extent, under the action of several stimuli (ibid.). Moreover, levels of brain S100B are elevated in the aging brain and in several pathological conditions such as Alzheimer disease, epilepsy and brain infectious diseases, as well as in Down syndrome in consequence of S100B's human gene mapping to chromosome 21q22.3. Thus, the concept has emerged that S100B might act an unconventional cytokine or a damage-associated molecular pattern protein playing a role in the pathophysiology of neurodegenerative disorders and inflammatory brain diseases. S100B's pro-inflammatory effects require relatively high concentrations of the protein, while at physiological concentrations S100B exerts trophic effects on neurons (ibid.). Most, if not all of the extracellular (trophic and toxic) effects of S100B in the brain are mediated by the engagement of RAGE (receptor for advanced glycation end products). for example, high S100B activates RAGE-dependently microglia, stimulating the release of IL-1β and TNF-α and upregulating the expression of the pro-inflammatory enzyme, COX-2 (ibid.). We show here that high S100B also chemoattracts microglia via RAGE engagement and RAGE-dependent activation of Src kinase, Ras, PI3K/Akt, RhoA/ROCK, Rac1/JNK/AP-1 and Rac1/NF-κB, with marginal roles for ERK1/2 and p38 MAPK. The S100B/RAGE-dependent activation of Ras/Rac1/JNK/AP-1, Ras/PI3K/Akt/NF-κB and Ras/Rac1/NF-κB results in the upregulation of expression of the chemokines, CCL3 and CCL5, the activity of which is required for S100B to chemoattract microglia. These results suggests that S100B might participate in the pathophysiology of brain inflammatory disorders via RAGE-dependent regulation of several inflammation-related events such as attraction and activation of microglia.

1736/B115

Collagen I and II Processing by MMPs Associated with Human Dendritic Cells.

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In recent years several lines of research indicate the relevance of extracellular matrix remodeling in tissue homeostasis. Several enzymes, including matrix metalloproteinases (MMPs), have been described as modulators of tissue microenvironment through degradation of the extracellular matrix (in particular collagen metabolism), processing of cytokines and growth factors and processing of cellular surface receptors. The MMPs are the primary enzymes involved in collagenolysis. Peptide fragments generated by collagen processing could be presented to the immune system for generation of either tolerance or immunity. Elucidating the molecular basis of collagen processing and MHC class II presentation of its peptides is critical for the understanding of the immunological response occurring in many inflammatory, autoimmune and degenerative diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and degenerative arthritis. Herein, we are presenting new evidences that collagen-I and -II were processed by a panel of pure recombinant MMPs (such as MMPs 1-3 and 6-13)) leading to the generation of new peptides epitopes (with molecular weights of 800-6,000 Da), which were sequenced using peptidomics-proteomics tools (such as LTQ MS/MS electrospray ionization mass spectrometry). In addition, new biochemical assays were developed to monitor collagen-I and -II processing by MMPs associated with the plasma membrane fraction isolated from human primary dendritic cells. In order to reveal the new MHC class II restricted epitopes processed by MMPs that are distinct and additional to peptides processed by endosomal proteases, we further developed biochemical assays and sequenced peptides derived from degradation of collagen-I and -II by the recombinant cathepsins S, B, D and L. Altogether, our new discovered epitopes suggest new
mechanisms for extracellular antigens processing which could be responsible for the onset of some autoimmune diseases.

1737/B116
Upregulation of the Membrane Type-1 MMP in Isoproterenol-induced Myocardial Fibrosis of the Lumican-null Mice.

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Cardiovascular disease is a leading cause of morbidity and mortality that occurs with increasing incidence in the elderly. With aging, the myocardium undergoes structural remodeling and fibrosis. An important component of structural remodeling is remodeling of the extracellular matrix (ECM). The intimal proteoglycan composition includes the class II small leucine-rich proteoglycans lumican, which was identified from a human placenta cDNA library by the expression cloning method as a gene product that interacts with membrane-type matrix metalloproteinase-1 (MT1-MMP) or MMP14. The determinants that lumican regulates cardiac fibrosis, remain poorly understood. The present study hypothesized that by the neurohormonal stimuli cardiac hypertrophy was a result of changes in ECM remodeling. Accordingly, the goal of this study was to examine the effects of aging on LV structure, collagen, MMP, and TIMP levels, and myocardial fibroblast function in Lumican-null mice. The unique findings of this study were as follows. First, the fragile basements of the Lumican-null mice were demonstrated. In the molecular level, brain natriuretic peptide, which secreting by the ventricles of the heart in response to excessive stretching of myocytes in the ventricles, was increased also. Second, MT1-MMP protein level was compensated increasedly by the neurohormonal stimuli. In conclusion, the lumican protein plays an important role in the processing of cardiac fibrosis.

Intermediate Filaments (1738 – 1742)

1738/B117
Cytokeratin 18 Is a Specific Marker of M Cell of Bovine Peyer's Patches.

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Microfold (M) cells are a kind of intestinal epithelial cell in the follicle-associated epithelium (FAE) of Peyer’s patches. M cells can transport antigens and microorganisms into the underlying lymphoid tissues and have an important role in mucosal immune responses. However, the origin, differentiation, and cell death mechanisms of M cells remain unclear, and a primary source of difficulty for investigations of bovine M cells is due to lack of their available specific molecular marker. To clarify a specific marker of bovine M cell, we investigated the expression of intermediate filament protein by immunohistochemistry using the Peyer’s patch of calf. Jejunal FAE was discontinuously immunostained with cytokeratin (CK) 18, and the continuous immunostaining of CK18 was observed in ileal FAE. A couple of mirror sections was used for immunostaining with anti-CK18 and anti-Ki-67, a proliferative cell marker. CK18 was expressed in Ki-67-positive proliferating cell of crypt. These staining patterns of CK18 were quite similar to the localization of M cells in FAE, which were confirmed by scanning electron microscopy (SEM). The ultrastructure of CK18-positive cells by SEM indicated that CK18-positive cells in FAE had irregular and sparse microvilli, as a typical morphological characteristic of M cells. In contrast, CK18-negative cells in FAE had regular and dense microvilli, like as absorptive enterocytes. CK20 was clearly observed in villous epithelial cells and CK18-negative cells in FAE, but not in crypt. These results revealed that the expression of CK 18 was detected in crypt of Peyer’s patches and continued in M cells of FAE, and that enterocytes changed CK18 for CK 20 during
The apicomplexan parasite Toxoplasma gondii divides asexually through a unique and poorly defined process of internal daughter budding. The physical infrastructure for this species-specific process is a dynamic cytoskeleton composed of microtubules, flattened vesicles (alveoli) and a family of intermediate filament proteins (TgIMCs). The latter two elements make up the inner membrane complex (IMC). We have identified 14 TgIMC proteins based on the presence of a repeat shared across the Avelolata, the alveolin-repeat. Previous to this project only TgIMC1, TgIMC3, and TgIMC4 had been studied. We have cloned and expressed all 14 proteins as YFP fusions to study their sub-cellular localization. Each TgIMC displays unique dynamics throughout development, but three physically distinct localizations were identified: eight TgIMCs localize to the alveoli, five TgIMCs localize to a mysterious structure known as the posterior cup, and TgIMC11 localizes to the apical cap in mature parasites as well as the IMC of daughter buds. Interestingly, some of the posterior cup localizing TgIMCs (TgIMC5, 8, 9 and 13) start associating with the basal complex halfway through the internal budding process and are first visible as a ring structure juxtaposed to TgMORN1. Upon completion of budding, these posterior TgIMCs form a multi-ring structure to create the posterior cup. TgIMC15 localizes strongly at the apical end in addition to the buds and posterior cup and appears to be an early marker of division. The variety of localization patterns observed suggest complex interactions between the TgIMCs themselves and other proteins involved in division. We are currently investigating these interactions through yeast two-hybrid assays. These new findings provide unprecedented detail into novel steps in the cell division process. We will discuss how we plan to dissect the control mechanisms underlying the surprisingly complex behavior of the alveolins throughout parasite division.
pellet completely if assembled alone. These results are interpreted in the context of the unique optical and mechanical properties of fish lenses.

1741/B120  
**Dynamic Network Formation of Vimentin Intermediate Filaments Revealed by Atomic Force Microscopy, Rheology and Mathematical Modeling.**  
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Intermediate filaments (IF) constitute one of the three principal cytoskeletal filament systems in metazoan cells. They are 10-nm in diameter and are made from fibrous proteins forming assembly-competent units consisting of 60-nm long, highly charged tetrameric complexes of anti-parallel, half-staggered coiled coils. Without need for nucleotides and other cofactors, IF proteins can be induced to assemble by a change in the ionic conditions. The first assembly product forming within seconds by lateral association of 8 tetramers is a 60-nm-long, full-width unit-length filament (ULF). The driving force for this association is the interaction between the highly basic non-alpha-helical head domains and the acidic alpha-helical rod domains. These ULFs are the basic units for filament elongation. The kinetics of filament formation can easily be controlled by the protein concentration and the ionic conditions, and the filament-length distribution can be determined by electron and atomic force microscopy. Besides their unique assembly mechanism, IFs differ further from microfilaments and microtubules by their unique ability to exhibit a substantial strain stiffening upon being mechanically stressed. This property makes them the prime cellular system to establish and regulate cell plasticity giving it a role often referred to as mechanical "stress absorber". for the mesenchymal IF protein vimentin, we have established a mathematical apparatus to describe the assembly process in quantitative terms. Furthermore, by classical rheology we have worked out the time-dependent networking properties of IFs, which are ruled by their persistence length and the mesh size. for vimentin, a persistence length of 0.4 micrometers and a mesh size of around 0.2 micrometers has been determined. These investigations have now been extended to mutated vimentin, either causing disease (lens cataract) or affecting assembly properties such as phospho-mimetic replacements of serines highly phosphorylated in cells during dynamic rearrangements of the vimentin network.

1742/B121  
**The Role of Vimentin in the Epithelial to Mesenchymal Transition (EMT): Cell Shape, Adhesion and Motility.**  
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Initiation of vimentin (V) expression is a canonical marker of the EMT during normal embryogenesis and in the metastatic conversion of primary tumor cells. Mammary duct epithelial cells (MCF-7) express keratin intermediate filaments (KIF) but not vimentin (VIF). VIF assembly can be rapidly induced in these cells following the microinjection of soluble vimentin. Coincident with VIF assembly, the cuboidal shapes of MCF-7 are altered into fibroblastic shapes within hrs, as determined by form factor analysis (FF; ctrl, 0.78 vs. V, 0.51, p<0.002). Similar results are obtained after inducing VIF by transfecting MCF-7 cells with vimentin cDNA; cells flatten, spread and elongate (FF; 0.67 vs. 0.44, p<0.002). Embryo fibroblasts harvested from vimentin-knockout mice (v/- mEF) are significantly more cuboidal in shape than normal mEF (mEF, 0.27; v/- 0.59, p=0.002) and the induction of VIF into v/- mEF induces mesenchymal shapes with a FF similar to that of normal mEF (V+, 0.32, p<0.002). Loss of VIF reduces mEF motility (mEF, 0.58 vs. v/- mEF 0.14 μm/min), while the scattering and motility of MCF-7 cells increases dramatically concurrent with VIF expression (scattering, MCF7 <3%, +V 48%; motility, ctrl, 0.24 vs. V+, 0.40
μm/min, p<0.002). Conversely, human fibroblasts expressing only VIF become more epithelioid following the knockdown of vimentin expression by shRNA or the disruption of VIF assembly by expression of a dominant-negative mutant vimentin (FF; shRNA, 0.49 vs. ctrls 0.35, p<0.002; vim1A: 0.56 vs. ctrls 0.26, p<0.002). Since focal adhesion dynamics increase in motile cells, we performed FRAP experiments on GFP-paxillin in VIF-expressing MCF-7 cells, and found a 400% increase in paxillin turnover, further emphasizing the role of VIF in cell motility. Results to date suggest that the different crosstalk in which VIF and KIF participate underlie these behavioral changes, and that VIF-mediated interactions may be dominant over those involving KIF, for example, MT depolymerization induces dramatic VIF network reorganization and cell shape change in VIF-containing cells, including MCF-7 cells containing VIF, whereas KIF-containing cells maintain normal KIF organization and cell shapes. Supported by NIGMS.

Actin Dynamics and Assembly II (1743 – 1763)

1743/B122
In Vitro Reconstitution and Protein Composition Analysis of Yeast Actin Patch-like Structures.
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Clathrin-mediated endocytosis in yeast requires the progressive assembly of more than 60 proteins at localized cortical sites. About half of these proteins are involved in the formation and regulation of a dense network of actin filaments which are nucleated by the Arp2/3 complex and provide the pulling force required for membrane invagination. In order to improve our understanding of how these actin structures are assembled, we attached one of the earliest known actin regulators to be recruited to endocytic sites, the WASP homologue Las17p, to polystyrene microbeads. When incubated in yeast extracts, these microbeads formed branched actin filament networks and some beads became motile. In order to identify the protein components of these structures, we purified them and analysed their composition by Multi dimensional Protein Identification Technology (MudPIT). We identified in the actin networks all the proteins known to belong to the actin module, together with amphiphysins. Several proteins from the coat protein module colocalize with Las17p at the surface of the beads, confirming their potential role in modulating actin assembly. Interestingly, we also found that the recruitment of actin binding proteins is selective. None of the proteins known to exclusively regulate the formation of actin cables or the actin contractile ring In Vivo were identified in the branched networks. Finally, this study enabled us to identify several new interactors, including some proteins recently discovered in eisosomes, PIK patches and TORC2 complexes, establishing intriguing biochemical interconnections between the different yeast cortical patches.

1744/B123
Arp2/3 Regulators in Actin Assembly and Endocytosis in Saccharomyces cerevisiae.
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The assembly of actin filaments, within dynamic, branched networks, provides the force for many types of cell motility. The proper regulation of Arp2/3 activity is likely to be critical for efficient generation of force and movement by actin networks, suggested in part by the presence of several potential Arp2/3 regulators in many cells. An outstanding question, essential for our understanding of this type of actin based motility, is how Arp2/3 regulators specifically influence the function of actin networks, and how the activities of several spatially and temporally colocalized regulators are coordinated in vivo. The yeast actin patch, which corresponds to sites of endocytosis, contains five conserved proteins that activate Arp2/3 in vitro. We previously found that Arp2/3 regulators have both distinct and overlapping roles in patch formation, the initiation of
movement away from the membrane, and the movement of the patch into the cytoplasm. However, it remains unclear the molecular mechanism by which Arp2/3 regulators are influencing the assembly of actin networks at patches. To better understand the mechanisms by which these Arp2/3 regulators influence network formation, we have developed techniques to monitor the composition of dendritic nucleation proteins within the patch, over time, using quantitative, live cell, fluorescent microscopy. Using a well-studied internal reference protein, we are able to determine the number of molecules of patch proteins throughout the lifetime of actin patches in living cells. From these studies we have determined the stoichiometry of many critical actin regulatory proteins within the patch, including Arp2/3, capping protein, Abp1, fimbrin/Sac6, WIP/Vrp1, cofilin, Aip1, coronin, Myo3 and Myo5. We have analyzed how the stoichiometry of the central dendritic nucleation proteins is altered in strains harboring mutations in the acidic/DDW regions of the Arp2/3 regulators WASp/Las17, Myo3 and Myo5. By understanding how the amount of actin, Arp2/3 and barbed ends are altered when these, and other, Arp2/3 regulators cannot bind to Arp2/3 we will gain insight into how they regulate actin network assembly in vivo.

1745/B124
Mechanism of Endocytic Actin Patch Assembly and Disassembly in Fission Yeast.
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Clathrin-dependent endocytosis in yeast depends on Arp2/3 complex-mediated actin assembly at endocytic sites into structures called actin patches. Previous work in budding and fission yeast defined the temporal order of assembly of endocytic patches. We used calibrated spinning disk confocal microscopy with internal standards to measure the number of molecules for 16 key endocytic patch components over the time course of assembly of individual patches. These measurements provide insights into the structure of the patch and constrain quantitative models of the mechanism of actin patch assembly and disassembly. The pathway begins 2 minutes before patch internalization with accumulation of ~40 clathrin molecules, sufficient to build a hemisphere but not a complete cage of clathrin around the endocytic vesicle. Clathrin is followed by precisely timed waves of accumulation of proteins that appear and reach characteristic peak numbers at specific times relative to the time of patch internalization. Endocytic adaptor proteins appear 30-40 s before internalization and reach peak of ~120 End4p and ~230 Pan1p molecules at internalization. Activators of Arp2/3 complex appear 12 s before internalization and accumulate peak ~200 Wsp1p, ~120 Vrp1p and ~340 Myo1p molecules 3 s before internalization. Arp2/3 complex appears 9 s before internalization and induces actin assembly that culminates in accumulation of peak ~300 Arp2/3 complexes, ~7,000 actins, ~900 fimbrins and ~200 capping proteins at internalization. These numbers show that the filaments are short and highly crosslinked. Upon patch internalization, the actin network disperses in 10 s. We simulated actin assembly and disassembly downstream of transient wave of NPF activation. Mathematical simulations revealed that dendritic nucleation model explains self-assembly and destruction of the actin network but the process requires faster binding of Arp2/3 complex to the side of mother filaments than observed In Vitro and severing filaments into oligomers that diffuse away rather than depolymerizing. The Arp2/3 complex inhibitor coronin, which arrives last and peaks 5 s after internalization, may cooperate with cofilin in network disassembly.

1746/B125
EGF Stimulation Triggers the Early Recruitment of Cofilin and Arp2/3 at the Leading Edge Prior to the First Barbed End Transient.
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Synergistic interaction between Cofilin and the Arp2/3 complex promotes local actin polymerization and protrusion in response to Epidermal Growth Factor (EGF) in MTLn3 cells. We have shown before that Arp2/3 and Cofilin are recruited to the leading edge within 1 minute after
EGF stimulation, within a lamellipod region wider than the barbed end distribution. We have here refined these kinetics by analysing the spatial and temporal recruitment of Arp2/3 and Cofilin at the edge within the first minute after EGF stimulation using live microscopy of cells expressing Arp3-GFP or Cofilin-GFP. We have identified a previously unknown step in early lamellipod extension, with an initial slow extension followed by a transient pause/retraction at 40 sec after stimulation, and rapid extension thereafter. This profile is matched by the polymerized actin levels in cells and at the leading edge, which peaks at 15 sec, transiently drops at 40 sec, and increases thereafter in a broad peak. Arp2/3 recruitment to the leading edge also follows 2 phases, with an early recruitment 35 secs after stimulation, within a region adjacent to the cell edge (starting 0.2 um from the membrane). Arp2/3 is later recruited with a wider distribution throughout the leading edge up to the membrane (starting at 40-45 sec after stimulation), in parallel with increased actin polymerization and lamellipod extension. In contrast, Cofilin is never recruited to the extreme edge of the cells. It starts accumulating progressively around 10 sec before Arp2/3, within a broad area (0.3-1 um from the cell edge), stalls around 40-45 sec and resuming at 60 sec, matching the polymerised actin kinetics. Overall our results suggests that EGF stimulation triggers an early influx of Cofilin and Arp2/3 at the leading edge, prior to the first barbed end transient, that helps prepare the actin network for efficient lamellipod extension through Arp2/3-mediated actin polymerisation at the membrane.

1747/B126
A Novel ADF/cofilin Homologue that Binds Arp2/3 Complex and Regulates the Nucleation and Turnover of Branched Actin Filaments.
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The Arp2/3 complex is a conserved actin-nucleating machine that produces branched filament networks and is harnessed to a wide range of cellular processes. Here, we identify Aim7/GMF (Altered Inheritance of Mitochondria/ Glia Maturation Factor) as a novel direct regulator of Arp2/3 complex. Aim7 is a structural homologue of ADF/cofilin and is conserved from yeast to humans; however, its function has until now remained unclear. We found that purified S. cerevisiae Aim7, unlike ADF/cofilin, lacks detectable affinity for G-actin, binds only very weakly to F-actin, and fails to promote F-actin severing and/or depolymerization. To better understand Aim7 function, we isolated its cellular binding partners by affinity chromatography and identified Arp2/3 complex and coronin. Co-immunoprecipitation experiments and binding assays further suggest that Aim7, coronin, and Arp2/3 complex form a ternary complex. To decipher the mechanism of action of Aim7, we used total internal reflection fluorescence (TIRF) microscopy to directly visualize real time dynamics of individual actin filaments in the presence of different mixtures of Arp2/3 complex, WASp-VCA, Aim7, and coronin. Very low concentrations of Aim7 (10 nM) dramatically accelerated debranching of filaments. Consistent with this branch turnover activity, aim7 null cells showed synthetic growth defects with cof1-22 cells in vivo. Higher concentrations of Aim7 (>100 nM) inhibited the nucleation of branched filaments by Arp2/3 complex and synergized with coronin in this function. Aim7 also localized In Vivo with coronin and Arp2/3 complex to cortical actin patches. Together, these observations suggest that Aim7 is a unique ADF/cofilin family member that binds to Arp2/3 complex and serves as a potent and multifunction regulator of branched actin network assembly and turnover.

1748/B127
A New Synergistic Mechanism for Promoting Nucleotide Exchange on Actin Monomers that Depends on ADF/cofilin and Srv2/CAP.
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In cellular processes fueled by rapid actin polymerization and turnover, nucleotide exchange (ATP for ADP) on actin monomers can become the rate-limiting step. However, the mechanisms for regulating nucleotide exchange on actin have remained poorly understood. Filament turnover induced by ADF/cofilin leads to a rapid accumulation of ADP-actin monomers bound to ADF/cofilin. This species is blocked from undergoing nucleotide exchange. Current thinking is that profilin functions to drive nucleotide exchange on actin. However, our direct tests show that profilin has almost no ability to promote nucleotide exchange on ADP-actin in the presence of ADF/cofilin. Thus, it is unclear what cellular factor might perform this function. Previously, it was reported that the conserved actin-binding protein Srv2/CAP could displace ADF/cofilin from actin monomers and thereby permit nucleotide exchange. Here, we extend these findings and report a striking and unexpected synergy between yeast Srv2/CAP and ADF/cofilin in catalyzing actin nucleotide exchange, which depends on the presence of both proteins. These effects require the WH2 domain of CAP, the function of which has been unknown. Purified Srv2/CAP WH2 domain binds directly to actin monomers, and point mutations abolish actin binding and disrupt full-length Srv2 synergy with ADF/cofilin in promoting nucleotide exchange. The same point mutations severely impair Srv2 In Vivo function in regulating actin organization, cell growth, and cell morphology. This work identifies Srv2 and ADF/cofilin as surprising co-factors in a new mechanism for driving nucleotide exchange on actin to promote turnover.

1749/B128
Single Molecule Study of VASP Reveals Molecular Basis for Actin Barbed End Polymerase Activity.
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Migrating epithelia and neuronal cells utilize the actin cytoskeleton to generate membrane protrusive forces. Of the many actin network architectures, these cells frequently build filopodial which provide a mode for cell-cell communication, formation of focal adhesions, and directional migration. Underlying filopodia formation are changes in the leading edge actin filament length distribution that favor the transition from a branched lamellipodia to a tightly bundled parallel unbranched network. This transition is regulated at least in part by actin nucleators controlling the branching frequency (e.g. Arp2/3 and formins) and the leading edge concentration of barbed end associated proteins (e.g. capping protein and the Ena/VASP protein family). Although the Ena/VASP protein family can antagonize capping protein and Arp2/3 dependent filament branching in vivo, it’s not clear how Ena/VASP regulates actin polymerization in vitro. To determine how Ena/VASP proteins regulate actin assembly we developed a way of visualizing single VASP tetramers in vitro. Visualization of single VASP tetramers revealed novel mechanisms regulating lateral F-actin interactions, barbed end association, and processivity. We believe our work provides new mechanistic detail about how Ena/VASP proteins regulate actin polymerization and network architecture in vivo.

1750/B129
High Affinity WH2 Domain/G-actin Interactions Reveal the Molecular Basis for Fast VASP-mediated Actin Assembly.
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Ena/VASP proteins are a conserved family of multi-domain actin regulators found in motile eukaryotes. They interact with actin via their WH2-like G-actin (GAB) and F-actin binding (FAB) sites located within their EVH2 domain and are able to accelerate actin assembly in vitro. Clustering of VASP to a surface triggers processive actin filament elongation even in the presence of capping protein (CP), suggesting that VASP acts as a powerful, membrane-anchored filament elongator at the tips of lamellipodia and filopodia. Remarkably, the Dictyostelium orthologue (DdVASP) enhances filament elongation 7-fold, whereas the three mammalian VASP, Mena and EVL accelerate elongation maximally 2-fold. To identify the molecular requirements for fast filament elongation, we designed chimeras by transplanting GAB and FAB of DdVASP either
alone or in combination into the backbone of human VASP (hVASP). As assessed by In Vitro TIRF microscopy, simultaneous insertion of the DdGAB and DdFAB into hVASP resulted in an equally high elongation rate as with DdVASP. The GAB motif, which represents a typical WH2 domain, turned out to have the most profound impact on filament elongation. Since the conserved GAB motifs from mammalian Ena/VASP members differ noticeably from the DdGAB, we assumed that the elongation rate depends on different actin-binding properties of the WH2 domain. We therefore determined the affinities of the GAB motifs for monomeric actin using analytical ultracentrifugation and show that the DdGAB binds to actin with an about 1000-fold higher affinity than hGAB. Therefore, we hypothesized that the filament elongation rate is directly correlated to the affinity of the GAB to G-actin. To confirm this, we constructed new hVASP chimeras encompassing previously characterized WH2 motifs from other actin-binding proteins with different affinities for G-actin. According to their affinities, all chimeras accelerated filament elongation to different extents both in solution and processively on beads. Collectively, our results provide strong experimental evidence for a general, affinity-based mechanism of WH2-mediated actin filament elongation that might be common for other WH2-containing proteins in sites of active actin assembly.

1751/B130
X-ray Crystal Structures and Molecular Mechanism of Improved Arp2/3 Complex Inhibitors.
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Arp2/3 complex regulates actin dynamics by nucleating branched filaments in response to cellular signals. Roles for the complex in leading edge protrusion, endocytosis, and formation of bacterial actin comet tails are well-established, but its function in other cellular processes has been difficult to establish due to a lack of simple and reversible methods to block Arp2/3 activity. Recently, we reported the characterization of two small molecules, CK-0944636 (CK-636) and CK-0993548 (CK-548), that directly bind to different sites on Arp2/3 complex and reversibly inhibit its activity. Altered versions of these parent compounds which are better Arp2/3 inhibitors In Vitro and In Vivo have been reported. We solved x-ray crystal structures of each of these new compounds, CK-0944666 (CK-666) and CK-0157869 (CK-869), bound to Arp2/3 complex to determine why they are better inhibitors. Like CK-548, CK-869 binds to a hydrophobic cleft in Arp3, causing a loop in subdomain 1 to flip into an open conformation. The ortho- and para-methoxy groups of CK-869 interact with the bottom of the loop, increasing the overall buried surface area of this inhibitor by 32 Å² compared to CK-548. Both CK-636 and CK-666 bind at the interface of Arp2 and Arp3, and are thought to lock the complex into an inactive conformation. In CK-666, thiophene is replaced by an ortho-fluorobenzene ring, which better fills the seam at the interface of Arp3 and Arp2 (16 Å² more buried surface area). We have initiated studies to determine the molecular mechanism of these inhibitors. An etheno-ATP binding assay shows that neither inhibitor affects ATP binding to Arp2/3 complex. In addition, we show here that binding of Arp2/3 complex to the pointed end of actin filaments is not affected by either inhibitor. Further biochemical characterization of these compounds will allow us to determine how they inhibit Arp2/3 complex, providing insight in to the molecular mechanisms of Arp2/3 complex regulation.

1752/B131
Identification and Characterization of a Small Molecular Inhibitor of Formin-Mediated Actin Assembly.
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Formins stimulate actin filament assembly for fundamental cellular processes including division, adhesion, establishing polarity and motility. Formin inhibitors would be extremely useful because most cells express multiple formins whose specific functions are not yet known, and because metastatic tumor formation depends upon the deregulation of several formin-dependent processes. We identified a small molecule inhibitor of formin homology 2 domains by screening compounds for their ability to prevent formin-mediated actin assembly in vitro. KV13 inhibits
formins from evolutionarily diverse organisms including yeast, nematode worm and mice, with a half-maximal inhibitor concentration of ~15 µM. Low micromolar concentrations of KV13 disrupts formin-dependent, but not Arp2/3 complex-dependent, actin cytoskeletal structures in fission yeast and mammalian NIH 3T3 fibroblasts. Therefore KV13 is an effective general inhibitor of formin-mediated actin assembly.

1753/B132
Biochemical and Cellular Functions of the Formin Protein, INF2.
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Formins are proteins that accelerate actin nucleation, then influence actin filament elongation rate by remaining at the barbed end. INF2 is a biochemically unique mammalian formin protein in that it accelerates both actin polymerization and depolymerization. In 3T3 fibroblasts, INF2 localizes to the endoplasmic reticulum (ER), with localization being relatively uniform throughout the ER. This localization depends on post-translational farnesylation at the C-terminus, but also upon the FH2 domain. Knock down of INF2 in fibroblasts does not alter ER morphology. We are currently investigating INF2 cellular function in more detail, including roles in vesicle transport and in cell death pathways. In addition, we are elucidating the mechanisms behind INF2’s unique depolymerization activity in more detail. One interesting result is that, while INF2’s actin depolymerization activity is auto-inhibited, its polymerization activity is not.

1754/B133
The Three Fission Yeast Formins Have Unique Actin Assembly Properties That May Be Tailored for Their Distinct Roles In Polarized Cell Growth.
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Fission yeast has three formins that each drive actin assembly for a distinct polarized structure: actin cables (For3), the contractile ring (Cdc12), and the mating projection tip (Fus1). We propose that both regulation (activation at the right time and place) and key actin assembly properties tailor each formin for its particular role. To begin to test our hypothesis, we report the first biochemical and biophysical characterization of the FH1FH2 actin assembly domains of the fission yeast formins For3 and Fus1, and compare their properties to the well-characterized formin Cdc12. We found that like most other formins including Cdc12, both For3 and Fus1 stimulate nucleation, remain processively associated with the elongating barbed-end in both the absence and presence of profilin, and accelerate the rate of profilin-actin addition to the barbed-end. However, both the nucleation efficiency and elongation rates vary markedly between the fission yeast formins. Cdc12 and Fus1 are highly efficient nucleators (~1 filament per 4 formins), whereas For3 is an extremely poor nucleator (~1 filament per 100 formins). Unlike most formins, in the absence of profilin all three fission yeast formins drastically slow the barbed-end elongation rate compared to the control rate of 10 sub s-1 µM-1 (Cdc12/For3 ~0.1 sub s-1 µM-1, Fus1 ~0.5 sub s-1 µM-1). All three formins accelerate barbed-end elongation in the presence of profilin but at different rates (Fus1 ~5 sub s-1 µM-1, Cdc12 ~10 sub s-1 µM-1, For3 ~12 sub s-1 µM-1). Furthermore, chimeric formin FH1FH2 constructs demonstrate that elongation rates are not strictly proportional to the number of profilin-binding regions in the FH1 domain. Therefore, the barbed-end associated FH2 domain plays a significant rate-limiting role in determining the specific actin assembly rate. Our work suggests that formins have carefully tailored actin assembly properties to carry out their different roles in cell polarity.

1755/B134
Rho-Kinase-Dependent Phosphorylation of the Diaphanous Autoregulatory Domain Enhances mDia2 Activation.
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We have previously shown that the RhoA effector, mDia2, regulates serum response factor (SRF)-dependent smooth muscle cell (SMC)-specific transcription by stimulating actin polymerization and myocardin-related transcription factor (MRTF) nuclear localization. While it is clear that RhoA activates mDia2 by disrupting an intramolecular interaction between the diaphanous autoregulatory domain (DAD) and the diaphanous inhibitory domain (DID), it is currently unknown whether additional signaling pathways regulate mDia2 activity. We have identified a conserved acidic region within the DID domain (E377/D388) that likely interacts with the DAD basic region. Mutation of either region in mDia2 attenuated the DID-DAD interaction and partially activated mDia2 as measured by an increase in actin polymerization and SM marker gene transcription in 10T1/2 cells. We now demonstrate that mDia2 is phosphorylated In Vivo and In Vitro by Rho-kinase at two conserved residues (T1061 and S1070) just C-terminal to the DAD basic region. DAD peptides containing phosphomimetic mutations at these residues (T1061E and S1070E) have a reduced affinity for the DID and when overexpressed in 10T1/2 cells, fail to activate endogenous mDia. The activity of full-length mDia2 was greatly enhanced in the presence of the T1061E/S1070E mutation as determined by an increase in actin polymerization, MRTF nuclear localization, and SMC-specific gene transcription. Interestingly, mDia2 containing the T1061E/S1070E mutation, or mutations within the acidic and basic regions, were activated to a much greater extent by active (L63) RhoA in comparison to Wt. Taken together, our biochemical and functional data suggest that phosphorylation of the mDia2 DAD by Rho-kinase enhances mDia2 function by destabilizing the DID-DAD interaction resulting in greater activation by RhoA. These results should have important implications on mDia2-mediated regulation of actin polymerization as well as on the control of MRTF-dependent transcription.

1756/B135
One of a KIND: The 2.3-Å Crystal Structure of a Human Spire-Formin Complex.
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The actin nucleators Spire and Cappuccino (Capu) together comprise a signaling apparatus crucial for normal oocyte development in the fruit fly Drosophila melanogaster. Recent reports have shown direct binding between Spire and Capu, or their mammalian homologs Spir1 and Fmn-2 [Quinlan et al. (2007), J. Cell Biol. 179: 117-128; Pechlivanis et al., J. Biol. Chem. (in press)]. These interactions require the kinase non-catalytic C-lobe (KIND) domain of Spire/Spir1 and the C-terminal tail of Capu/Fmn-2. In this study, we determined the 2.3-Å crystal structure of the human Spir1 KIND domain in a complex with a portion of the human Fmn-2 C-terminal tail. The KIND domain adopts a fold largely similar to that of the C-lobe of protein kinases, as was previously predicted based on sequence homology. The Fmn-2 tail binds in a wide channel on the base of the domain through an interface composed of polar contacts. Comparisons to the structure of the C-lobe of p21-activated kinase-1 (PAK1) reveal that the KIND domain lacks an α helix in the position corresponding to the highly-conserved cG of the kinase fold. We propose that the KIND domain evolved as a protein-protein interaction module via deletion of this helix and its accomodation by a helical segment of its binding partner. This structure provides a novel paradigm for protein-protein interactions mediated through the KIND domain.

1757/B136
Probing the Mechanism of Actin Filament Bundling by the Formin, FRL2.
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Formins are proteins that accelerate actin nucleation, and subsequently influence the elongation rate of the newly nucleated filament. The formin homology 2 (FH2) domain is sufficient for these activities. The FH2 domain is a dimer that binds to and moves processively with the elongating actin filament barbed end. FRL2 is a mammalian formin that possesses an additional biochemical activity: it bundles filaments. The FH2 domain is sufficient for bundling. We have proposed a two-step strategy for bundling, whereby: 1) the FH2 binds the side of an actin filament by dissociation of the dimer, followed by re-association around the filament; then 2) bundling occurs when
residues on the outside of the FH2 dimer bind to a second actin filament. We are testing this bundling mechanism using a mutagenesis strategy. We derived a model of FRL2's FH2 domain from the crystal structure of another mammalian FH2 domain, that of DAAM1. from this structure, we chose residues predicted to mediate dimerization or interaction with additional actin filaments. We have identified several interesting mutants from this process.

1758/B137
Probing the Mechanism of Filopodia Assembly by the Formin, FRL2.
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Formins are proteins that accelerate actin nucleation, and subsequently influence the elongation rate of the newly nucleated filament. The formin homology 2 (FH2) domain is sufficient for these activities. The FH2 domain is a dimer that binds to and moves processively with the elongating actin filament barbed end. The FH1 domain binds the actin monomer-binding protein, profilin, and allows rapid elongation of profilin-bound actin at FH2-bound barbed ends. FRL2 is a mammalian formin that possesses an additional biochemical activity: it bundles filaments. The FH2 domain is sufficient for bundling. FRL2 constructs cause assembly of abundant filopodia and microvilli in multiple cell types, and both the FH1 and FH2 domains are required for this activity. We find similar results with the FH1-FH2 domain construct from mDia2, another bundling formin. Surprisingly, the FH1-FH2 domain of FRL1 does not cause filopodia/microvilli assembly, despite being able to bundle filaments In Vitro and being highly similar to FRL2. Chimeric constructs between FRL1 and FRL2 show that FRL2's FH2 domain is required for filopodia/microvilli assembly, while the FH1 domains of FRL1 or FRL2 are interchangeable for this activity. This finding leads us to conclude that FRL2's FH2 domain contains a motif that allows plasma membrane binding.

1759/B138
A Dual Screen Identifies Clathrin as a Novel Regulator of Lamellipodia Formation Independently of Its Established Role in Vesicular Transport.
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Lamellipodia are critical membrane structures for cell spreading and migration. The Scar/Wave Complex is an essential molecular machine for the formation of these structures. This machine alternates between an inactive conformation in the cytosol and an active conformation at the plasma membrane, which generates a branched actin network through activation of the Arp2/3 complex. In order to identify novel factors controlling this activation cycle, we performed a dual screen in Drosophila cells. Proteomics yielded potential partners of the complex and functional genomics yielded genes that gave the Scar/Wave phenotype upon RNAi mediated inactivation. The intersection identified surprisingly clathrin, a coat protein involved in budding membranes during endocytosis and vesicular transport, as a Scar/Wave regulator. Clathrin interacts with the Scar/Wave complex in both fly and human cells. This interaction is regulated by treatments that potentiate or impair lamellipodia formation. Using depletion of adaptor complexes or expression of a specific clathrin mutant, we were able to uncouple the classical role of clathrin from its novel role in lamellipodia formation. Upon clathrin depletion, the defect in lamellipodia formation was associated with a defect in Scar/Wave recruitment to the membrane. Conversely, artificial membrane targeting of clathrin enriched Scar/Wave at the membrane and potentiated cell spreading. These data establish a novel role of clathrin in the activation cycle of the Scar/Wave complex at the step of membrane recruitment. Thus clathrin, being a major player in endocytosis and in Scar/Wave-mediated actin polymerisation, is likely to control the balance between membrane retrieval and projection.
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Recently, membrane bridges between cells have emerged as a key cellular structure in intercellular communications; however, their dynamics is not understood. In this study, we demonstrated the formation and regulation of novel membrane-based ultrathin fibers in NIH3T3 cells using confocal microscopy and atomic force microscopy (AFM). Ultrathin fibers were formed in the adjacent region of neighboring cell by phorbol 12-myristate 13-acetate (PMA) and the fiber formation was prevented by Trolox, the reactive oxygen species (ROS) scavenger. In addition, glucose oxidase stimulated the formation of the ultrathin fibers, indicating the role of ROS in the fiber formation. The dimension of the ultrathin fibers was determined to be from 50 to 120 nm. The formation of PMA-induced ultrathin fibers was inhibited by cytochalasin D, the microfilament destabilizer, whereas taxol (the microtubule stabilizer) or colchicine (the microtubule destabilizer) did not show significant effect, indicating that ultra-thin fibers are mainly composed of microfilaments. Thus, we propose formation and regulation of novel microfilament-based ultrathin fibers with ultrastructural imaging, which may contribute to understanding diverse mechanisms of cell-to-cell communications and to revealing intercellular transfer of proteins or organelles.

Osmotic Swelling Induces the Differential Cytoskeleton Remodeling Between Normal and Cancer Cells.
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Hypotonicity-induced cell swelling is characterized by a modulation of cytoskeletal architecture and activation of membrane ion transport, which results in regulatory volume decrease. Little is known about the differential signal transductions involved in volume-regulated cytoskeletal dynamics of normal and cancer cells. This study aims to elucidate the dynamics of F-actin and actin-associated proteins in osmotic swelling of normal and cancer cells. In response to hypotonicity, cancer cells swelled and a profound alteration in cellular architecture occurred. The actin cytoskeleton was remodeled to counteract cell swelling, which consisted of the reduction of the ventral stress fibers and the formation of F-actin protrusions at the cell surface. Ezrin, a member of ERM family was concentrated in F-actin-enriched membrane protrusions, compared with cells in the isotonic condition, where ezrin had a typical localization in the cytosol. Hypotonicity also rapidly and robustly stimulated endogenous focal adhesion kinase (FAK) phosphorylation at Tyr-397 that colocalized with ezrin at F-actin-enriched membrane protrusions. More importantly, evidences from pixel-by-pixel image analyses and immunoprecipitation indicated that membrane recruitment of ezrin interacted with KCl cotransporter-3, a critical ion transport system involved in regulatory volume decrease. The converse alterations for actin network and ezrin recruitment were observed in parallel with regulatory volume decrease. More importantly, normal epithelial cells of cervix and ovary exhibited the different dynamics in F-actin remodeling and recruitment of actin-associated proteins in response to hypotonicity. Thus, the processes of osmo-sensitive cytoskeletal remodeling may provide the therapeutic intervention.

Cross-talk between the actin DNase-I Binding Loop, the Hydrophobic Loop and the Nucleotide Binding Cleft.
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Our goal was to shed light on the conformational cross-talk between actin’s DNase I-binding loop (D), the hydrophobic loop (H) and the nucleotide binding (NB) cleft, and to assess the role of their coupling in actin filament formation and stability. To this end, we examined the following: (i), site specific limited proteolysis of subdomain 2 (within the D-loop) by subtilisin and ECP; (ii), intramolecular disulfide cross-linking of the H-loop in (LC)2CA yeast G-actin; (iii), the interaction/mobility of EPR spin probes in WT, Q41C, S265C and (LC)2CA yeast actin mutants, and (iii), the rate of epsilon ATP insertion into the uncross-linked and disulfide cross-linked (LC)2CA G-actin. We found that subtilisin cleavage of the D-loop increased the mobility of spin probes attached to the H-loop in the S265C and (LC)2CA actin mutants, and to C374 in WT actin, indicating intra/intermolecular dynamic coupling between these loops and the C-terminus. Locking the H-loop to actin surface by a disulfide bridge in (LC)2CA actin inhibits ATP binding at the nucleotide binding site and leads to the release of epsilon ATP from the NB-cleft. This suggests signal propagation from the D-/H-loops to the NB cleft. Notably, the rate of ECP cleavage of disulfide cross-linked (LC)2CA G-actin is higher than that of uncross-linked actin, confirming intramolecular coupling between the H- and D-loops. These findings and the EM observations show the importance of dynamic connections among actin’s structural elements to the filament formation and stability.

1763/B142
**Mitochondria are Anchored by Actin Filaments to the Cortex and Are Motile in Sea Urchin Eggs.**
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We studied whether cellular organelles are freely floating in the cytosol or stabilized by binding to other structures by using centrifugal force. Unfertilized Arbacia punctulata eggs were centrifuged in order to stratify cellular organelles. Mitochondria, as visualized by staining with MitoTracker, formed a broad band in the mid region of the eggs. However, in the presence of latrunculin-A (Lat-A) which induces depolymerization of actin, they formed a narrow band upon centrifugation suggesting that actin filaments are involved in anchoring mitochondria possibly to the cell cortex. Similar behavior of mitochondria upon centrifugation was observed in either Ca-ionophore-activated unfertilized eggs or fertilized eggs too. but in the case of fertilized eggs both Lat-A and nocodazole were required to form the narrow mitochondrial band in a centrifuge tube. These results were confirmed by transmission electron microscopy. In live uncentrifuged eggs, both unfertilized and fertilized, mitochondria were localized near the cortex and quickly moved around. The movement was abolished or diminished by the addition of Lat-A. These results suggest that mitochondria are anchored to the cortex of sea urchin eggs either unfertilized or fertilized by actin filaments and undergo actin-dependent movement.

**Muscle Biochemistry and Cell Biology (1764 – 1784)**

1764/B143
**Phosphorylation of Tmod1 Thr54 Prevents Efficient Assembly at Thin Filament Pointed Ends.**
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Tropomodulin1 (Tmod1), an actin filament capping protein, plays an important role in thin filament length regulation by preventing actin polymerization and depolymerization at the pointed (slow-growing) ends in striated muscle. Recently, Ser2 and Thr54 within Tmod1 were identified as phosphorylation sites In Vitro for the TRPM7 (transient receptor potential cation channel,
subfamily M, member 7) kinase using the N-terminal fragment of Tmod1 (aa 1-92). In this study, we identified a third Tmod1 phosphorylation site (Ser163) using full-length Tmod1. Tmod1 Ser 163 is also phosphorylated In Vitro by TRPM7 kinase. To learn about the potential functional significance of the Tmod1’s phosphorylation sites, we generated phosphorylation-off [S2A T54A S163A] and single, double and triple phosphorylation-mimic [S2D T54E S163D] Tmod1 mutants and introduced them into neonatal rat cardiac myocytes. Interestingly, the assembly of GFP-Tmod1 was perturbed when the triple phosphorylation-mimic Tmod1 [S2D T54E S163D] was expressed. Further analysis with the single mutants revealed that Tmod1 Thr54 is critical for pointed end assembly. Surprisingly, however, over-expression of GFP-Tmod1 [T54E] resulted in a significant reduction in the thin filament lengths, similar to over-expression of WT GFP-Tmod1; this suggests that although GFP-Tmod1 [T54E] does not assemble well, it can still regulate thin filament lengths. on the other hand, when the triple phosphorylation-off Tmod1 [S2A T54A S163A] mutant was overexpressed, thin filament lengths were not altered, although this triple mutant assembled at the pointed ends of thin filaments. Taken together, our data suggest that pointed end assembly and at least one of Tmod1’s thin filament length regulatory mechanisms are not linked and that these two mechanisms are regulated by the phosphorylation propensity of Tmod1.

1765/B144
Automated Analysis of Skeletal Muscle Fiber Size and Metabolic Type.
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The accurate quantification of skeletal muscle fiber size and metabolic type is desired in studies in a variety of biomedical contexts including muscle regeneration, muscular dystrophy, exercise physiology, space flight, and nutrition. Our goal is to develop readily accessible automated image analysis algorithms to accomplish this task. To aid in development of the algorithms, muscle samples were collected from mouse, pig, and monkeys, and labeled for laminin and different myosin isoforms. The samples were then photographed via digital fluorescence microscopy. Algorithms were developed to identify the laminin label, segment the image into the individual fibers for quantification of fiber size, and apply the segmentation to the myosin image for quantification of fiber metabolic subtype. The algorithms were incorporated into the CyteSeer image analysis program for routine use of the algorithms on standard digital microscope platforms. Results from manual analysis were compared to those obtained from the image analysis algorithms. Estimates of fiber size for mouse gastrocnemius muscle were 1978 ± 565 micron2/fiber vs. 1970 ± 521, manual vs. automated analysis (mean ± SD, n= 4 samples, 29 to 87 fibers/sample), with an average % error = 0.12%. Very close agreement was also obtained for pig EDL muscle (5618 ± 987 vs. 5628 ± 967, 28 to 49 fibers/sample, n= 8 samples, average error = 0.25%). Regarding muscle fiber subtype identification, results were 53% vs. 51% for slow myosin in mouse soleus muscle, 11.1 % v. 10.7% for Fast Ila in swine EDL, and 20.8 vs. 20.3 % for slow myosin in monkey VL muscle. Automated analysis required approx. 5 seconds/image vs. 1 - 2 hours per image for manual analysis. The results demonstrate that algorithms can be developed for automated quantification of skeletal muscle fiber type that perform equivalent to manual quantification, and the algorithms greatly reduce the time required for such analysis.

1766/B145
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The sliding filament theory states that force generation by striated muscle sarcomeres is directly proportional to the degree of overlap among myosin thick filaments and actin thin filaments. Therefore, thin filament length is a critical determinant of force generation, but the interplay among thin filament-associated proteins in determining filament length is poorly understood. Nebulin coextends with actin along most of the thin filament, while a nebulin-free pointed-end extension is capped by tropomodulin (Tmod). Tropomyosin (TM) regulatory molecules also extend along the entire thin filament, binding to Tmod at the pointed end; Tmod-TM binding is proposed to enhance Tmods' capping affinity. We used a Tmod1-knockout mouse model to show that differential interaction affinities among TM and Tmod isoforms correlate with changes in thin filament length and force production in skeletal muscle. In the absence of Tmod1, embryonic skeletal muscles have longer thin filaments that are capped by Tmod4. In contrast, postnatal skeletal muscles lacking Tmod1 exhibit shortened thin filament lengths, pointed-end capping by Tmod4, and reduced isometric force generation. The absence of Tmod1 does not affect the organization of nebulin and other sarcomeric components, indicating that the observed decrease in force production in Tmod1-deficient skeletal muscle is not caused by disruption of myofibrillar structure. Additionally, Tmod4 binds to muscle TMs with a lower interaction affinity than Tmod1, providing a mechanism to explain the observed changes in thin filament length. Together, these data provide evidence that the coordinated action of a set of proteins, which includes nebulin, Tmods, and TMs, determines the particular thin filament length within an individual muscle. Therefore, specific combinations of these protein isoforms may optimize various muscles for their unique physiological functions.

**1767/B146**

**The Role of Telethonin C-terminus in de Novo Sarcomere Assembly.**

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Telethonin is found exclusively in striated muscle, where it tethers together N-termini of two titin molecules. The kinase domain of titin (TK) phosphorylates the C-terminal end of telethonin and this phosphorylation might be important in regulating the interaction of both proteins in the sarcomere. While some studies suggest that telethonin is dispensable for sarcomere assembly, truncation mutations in telethonin are associated with a form of autosomal recessive limb-girdle muscular dystrophy type 2G (LGMD 2G). We used anti-sense morpholino (MO+) technology to knockdown telethonin expression in Xenopus embryos. MO+ embryos are paralyzed and show severe sarcomeric disruption when compared to control embryos. In whole embryos and cell cultures stained for myosin or actin, very little or no de novo sarcomere formation is observed. Injecting exogenous full-length telethonin mRNA (FL: 1-176) in the MO+ background restores motility and myofibrillogenesis. Several C-terminal mutant constructs were then tested for rescue: 1) a full truncation of the phosphorylatable region (C’del: 1-163) and different telethonin derivatives encoding mutations in the C-terminal region such that it can no longer be phosphorylated 2) C’Ala and 3) C’mis. Rescue experiments with the C’del and C’Ala in the MO+ background do not relieve the paralytic phenotype or restore sarcomeric organization. However, the C’mis rescues at early stages followed by rapid and progressive degeneration of the sarcomeric structure. This result is particularly interesting since it appears to mimic LGMD 2G in myocytes. Our results show that the C-terminus of telethonin is required for sarcomeric organization and integrity.

**1768/B147**

**Titin’s Role in Sarcomere Assembly and Dynamics: A Live Imaging Approach.**

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Titin is a 3.7 MDa protein that spans the half-sarcomere and forms an elastic scaffold along the myofiber. Its multiple protein binding sites and data obtained in animal models suggest that titin acts as a template in sarcomere assembly and helps to maintain sarcomere structure in health and disease. Titin integrates into the Z-disc and M-band during sarcomere assembly and has
been proposed to coordinate the assembly of thick filaments and accommodate shear forces to provide mechanical stability. Our objective is to uncover the molecular mechanisms underlying sarcomere assembly and turnover. Therefore we have generated a knockin mouse model, in which we inserted the red fluorescence protein DsRed close to titin’s N-terminus at the Z-disc. Mice that are heterozygous or homozygous for the DsRed-insertion are phenotypically normal with normal development and fertility. This makes the Titin-DsRed animals a suitable model to study sarcomere assembly and disassembly. We have verified the model using fluorescence microscopy to visualize DsRed in primary cardiomyocytes and tissue sections of heart and quadriceps: the red fluorescence produces a striated pattern documenting the proper integration of titin into the sarcomere. The localization of DsRed was confirmed by co-staining with the Z-disc marker alpha-actinin. With our novel mouse model we are now able to visualize titin’s integration into the sarcomere in real time using life microscopy. Furthermore, we will use photobleaching to analyze protein turnover as well as longitudinal and lateral mobility of titin along the sarcomere in vivo.

1769/B148 
**PAT-4/ILK Is Required for Association of UNC-112/Kindlin to the Cytoplasmic Tail of PAT-3 Beta-Integrin in C. elegans Muscle.**

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Myofilaments within *C. elegans* body wall muscle cells are organized and anchored to the muscle cell membrane by dense bodies and M-lines. These structures are both analogous and homologous to vertebrate focal adhesion plaques. To clarify the molecular mechanisms regulating these integrin mediated attachment structures, we are investigating protein-protein interactions between integrin-associated proteins using a two hybrid approach. From yeast two hybrid screening with PAT-3 Beta-integrin cytoplasmic tail, we identified UNC-112/Kindlin, a FERM family protein located at dense bodies and M-lines. From the data of domain mapping experiments, we showed that binding of UNC-112 to PAT-3 cytoplasmic tail requires full-length UNC-112. We confirmed this interaction by GST-PAT-3 cytoplasmic tail pulldown of UNC-112 from a yeast lysate expressing HA-tagged UNC-112 or native UNC-112 from a *C. elegans* lysate. As reported previously, PAT-4/ILK is required for UNC-112 localization in vivo. We asked whether PAT-4 affects UNC-112 binding to PAT-3 the cytoplasmic tail. We found that co-expression of myc-PAT-4 in yeast with HA-tagged UNC-112 enhances binding of HA-UNC-112 to GST-PAT-3. Next, we addressed the molecular mechanism by which PAT-4 is required for UNC-112 binding to PAT-3. We obtained an insight by finding that when the UNC-112 C terminal half was used as a bait to screen a yeast 2 hybrid library, all the prey clones represented cDNAs for the N-terminal half of UNC-112. We have confirmed the interaction between UNC-112 N-terminus and UNC-112 C-terminus using purified proteins. We already reported that the UNC-112 N-terminal half binds to PAT-4. This interaction suggests a conformational change of UNC-112 between closed and open states. We hypothesize that this UNC-112 conformational change might be regulated by binding of PAT-4 to UNC-112 N-terminus, resulting in ability to bind to the PAT-3 cytoplasmic tail. Using purified proteins we observed a competition between UNC-112 C-terminal half and PAT-4 for binding to UNC-112 N-terminal half, supporting our hypothesis.

1770/B149 
**UIG-1, a Cdc42 GEF, Is Phosphorylated by the Giant Protein Kinase, TTN-1, in C. elegans Striated Muscle.**

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The muscle sarcomere contains a number of huge polypeptides (700,000 Da—4 MDa) composed of multiple Ig and Fn domains, one or two protein kinase domains, and in some cases, elastic regions. Whereas much is known about the function of vertebrate titin, much less is known about the other giant proteins. *C. elegans* striated muscle contains 3 giant proteins: twitchin, UNC-89 and TTN-1. TTN-1, is predicted to be 2.2 MDa, and located in the I-band (Flaherty et al. 2002).
TTN-1 can be viewed as a hybrid between invertebrate twitchin and vertebrate titin. Using the Ig25-Fn31-kinase region of TTN-1 as bait to screen a yeast 2-hybrid "bookshelf" of 23 known components of nematode muscle focal adhesions (M-lines and dense bodies), we identified UIG-1. UIG-1 is a binding partner for UNC-112 (Kindlin), is 919 residues in length, and contains both DH and PH domains (Hikita et al. 2005). UIG-1 is located at muscle dense bodies, and is a guanine nucleotide exchange factor (GEF) specifically for Cdc42 in vitro. By 2-hybrid assays, only the kinase domain of TTN-1 is required for interaction with UIG-1, whereas residues 188-919 of UIG-1 are required for interaction with TTN-1 kinase. The interaction has been verified by far western assay. By immunofluorescence microscopy, antibodies to TTN-1 and UIG-1 partially co-localize in worm sarcomeres. Under kinase assay conditions, TTN-1 Ig-Fn3-kinase phosphorylates UIG-1 in vitro. Kinase assays on deletion derivatives of UIG-1 show that phosphorylation occurs outside the DH-PH region, especially between residues 1-103, and to a lesser extent between residues 533-823. Within the region 1-103, we have determined that the primary site of phosphorylation is serine 97 (S97), with a secondary site at serine 96 (S96). Two mutant forms of UIG-1 (1-532) have been prepared: A96A97 (non-phosphorylatable), and D96D97 (phosphomimetic). By 2-hybrid assay, S96S97 (wild type) and A96A97 can bind, but D96D97 cannot bind to UNC-112. This suggests that phosphorylation of UIG-1 in this region normally inhibits interaction with UNC-112. We are currently testing the ability of these mutant forms to localize to dense bodies in transgenic animals, and whether the mutants affect Cdc42 specific GEF activity.

1771/B150
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ε-Sarcoglycan (SG) is a homologue of α-SG that is encoded in the causative gene for autosomal recessive limb-girdle muscular dystrophy (LGMD2D). Unlike α-SG, ε-SG is expressed in a variety of tissues and its loss-of-function mutations cause autosomal dominant myoclonus-dystonia syndrome (M-D), but not muscular dystrophy. We previously indicated that upregulation of the ε-SG gene in striated muscle ameliorates muscular dystrophy of mouse model by compensation of α-SG function. Since the ε-SG gene is a maternal imprinting gene, interference of the negative gene regulation might be one of approaches for therapeutic treatment of LGMD2D as well as M-D. In order to realize this idea, it is important to search cells that produce loss of imprinting (LOI) of the ε-SG gene, and investigate mechanism of LOI. A weak ε-SG expression of maternal allele has been reported by the analysis of uniparental disomy lymphoblastoid cell lines, suggesting that LOI of the ε-SG gene occurs in a part of blood cells. Thus, we examined allele-specific expression of the gene in the cells using mice (EtKO) that have a retroviral exon trap insertion. PCR analysis of peripheral blood leukocytes of heterozygous EtKO mice clearly showed ε-SG mRNA signal from paternal allele while maternal allelic signal was undetectable. The same expression pattern was observed in the analyses of spleen and bone marrow, but faint signals appeared under higher sensitive PCR condition. This finding suggest that LOI of the ε-SG gene might occur in a quite small number of blood cells although further analyses are needed.

1772/B151
Molecular Model for the Cooperative Activation of Molluscan Muscle by Calcium.
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The purpose of this study is to model the cooperative activation of molluscan muscle. Current models for calcium activation are based on three positions of tropomyosin, i.e., troponin-dependent (B), central (C), and myosin-dependent (M). Because molluscan muscle lacks troponin, the transitions of tropomyosin between positions C and M may be the basis for
cooperative activation of all filamentous myosin systems. We describe a basis for activation by which calcium binding non-cooperatively to the regulatory light chains of myosin regulates the affinity of myosin for actin, which results in a cooperative movement of tropomyosin to position M. We show that our model fits the calcium-dependent isometric force relationship of the striated adductor muscle of the scallop (R. M. Simmons and A. G. Szent-Gyorgyi, 1985, J. Physiol. 358: 47-64) given a calcium binding constant of 1 micromolar. The results suggest that myosin binding couples energetically to a conformational change in tropomyosin that propagates in position M. Expansion of segments of tropomyosin in position M promotes the association of uncoupled myosin, which stabilizes one coupled myosin for each segment. Exponential growth of segments by this feed-forward mechanism is limited by the finite supply of tropomyosin in position C. Although coupling is expected to increase the dwell time of one of the bound myosin per segment, the uncoupled myosin bound in a segment are energetically free of the regulation mechanism and capable of unrestrained cycling and force generation. A derivation of the model for molluscan muscle and its application to thin filament regulation may be found on-line (www.westga.edu/STEMresearch). This work was supported by NSF grant MCB-0508203.

1773/B152
FHL1-Null Mice Exhibit a Scapuloperoneal Myopathy-Like Phenotype, which Is Coupled to Defects in Muscle Fiber Oxidative Capacity and Function.
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Four-and-a-half LIM domain protein 1 (FHL1) is a member of the gene family encoding LIM domain-containing proteins. Recently, point mutations in FHL1 have been identified in human patients suffering from rare forms of muscular dystrophy, including X-linked scapuloperoneal myopathy (SMD) and dominant-negative reducing body myopathy. To understand the functional role of FHL1 in the development of these myopathies, we generated a mouse model in which all isoforms of FHL1 are ubiquitously ablated (FHL1-null). Our results demonstrate that FHL1-null mice develop a nonlethal and inheritable myopathy that mimics some aspects of the milder SMD phenotype observed in human patients, which is characterized by weakness and atrophy in the postural muscles and neck but hypertrophy in the shoulder girdle and arms. Changes in fiber type and excessive variability in fiber size were observed to varying degrees in different muscles extracted from limbs and neck of 9-month-old FHL1-null mice. Both NADH dehydrogenase staining and electrophoretic separation of MHC isoforms revealed an enrichment of type-1 (oxidative) fibers and a decrease in type-2 (glycolytic) fibers in FHL1-null muscles. Interestingly, centralized nuclei were only detected in type-1 fibers, demonstrating that muscle remodeling in FHL1-null mice is predominantly found in muscles with high oxidative capacity. Electron microscopy revealed that FHL1-null muscle fibers contained areas with abnormally large and densely populated mitochondria. To ascertain the functional consequences of these changes, physiological analyses were performed on FHL1-null muscles. Isometric contractile testing of the 5th toe EDL in 1- and 9-month old mice revealed an age-dependent decrease in isometric stress production coupled with shorter fiber length in FHL1-null mice. A treadmill exercise protocol performed on 1-year-old mice demonstrated that FHL1-null mice were also prone to low exercise endurance. Taken together, these data highlight an important role for FHL1 in skeletal muscle growth and homeostasis in vivo. We also demonstrate that genetic deletion of FHL1 leads to an inheritable SMD-like myopathy that is coupled with alterations in skeletal muscle oxidative capacity and function.

1774/B153
New Monoclonal Antibodies against the C-terminal M10 Domain of Titin.
Our aim was to develop antibodies against the C-terminal M10 domain of titin, a giant modular protein of striated muscle sarcomeres. Single titin molecules span from the sarcomeric Z-disc to M-band, forming a continuous filament system in the myofibrils. The C-terminal part of titin, residing in the M-band, comprises a kinase domain and ten Ig domains M1-M10, interspersed by the unique regions is1-is7. M-band titin has structural and signalling functions and is associated with several types of muscle disease. Mutations in the extreme C-terminus of titin underlie tibial muscular dystrophy (TMD) and limb-girdle muscular dystrophy 2J (LGMD2J). TMD is inherited dominantly, while in homozygotes the same mutations lead to LGMD2J. Most of the TMD/LGMD2J mutations are situated in titin’s last domain M10, but different lines of evidence suggest that they ultimately lead to a major structural change or cleavage of the entire titin C-terminus. Lack of a specific antibody for the M10 domain has, however, so far hindered detailed studies on the fate of mutant titin. We have now had monoclonal antibodies generated against human M10. Based on preliminary analysis by ELISA, western blotting (WB) and immunofluorescence (IF) microscopy, three clones (7-4-4, 11-4-3, and 14-2-7) were selected for production and have now been characterized in more detail. Epitope mapping on a peptide array showed that all the three antibodies recognize different linear epitopes in human M10 and show some reactivity towards the homologous mouse sequences. Western blotting of titin constructs confirmed that all the antibodies work in WB and helped to further define the epitopes. 7-4-4 recognizes the last amino acids of the entire titin protein, including the carboxyl terminus. 14-2-7 binds the wild-type sequence at the site of the Finnish major mutation, suggesting use as an allele-specific antibody. In IF, 11-4-3 and 7-4-4 produce M-band staining on human muscle cryosections and seem promising for microscopic studies, but further optimization of staining conditions is required. The novel antibodies are needed for pathogenetic studies on TMD/LGMD2J, and for basic research on the structure and function of the sarcomeric M-band.

1775/B154
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Mutations in the C. elegans gene unc-100 show reduced motility and disorganization of myofibrils. unc-100 was identified in a motility-requiring selection and polarized light screen for defects in muscle function and structure (Zengel & Epstein, 1980). Currently, two alleles are available for unc-100, su115 and su149. Through the use of a “swimming” assay, we determined that both alleles are slower moving than wild type, with su149 showing the most dramatic defect—su149 L4 or adults are ~65% slower than wild type. Brood sizes of each mutant allele are reduced by 50% as compared to wild type. The somatic gonad shows an abnormal morphology compatible with a defect in distal tip cell migration, although the number of distal tip cells is normal. By polarized light microscopy, pharyngeal muscle appears normal, but the body wall muscle cells have myofibrils with an overall reduction in birefringence and disorganization in the patterning of a and I-bands. The polarized light defect of su115, but not su149, is suppressed by growth at lower temperature (15o C). Electron microscopy shows missing M-lines, broken dense bodies that are often detached from the cell membrane, and a lack of defined a and I bands. Staining of unc-100 mutant muscle with antibodies to a variety of known sarcomeric proteins reveals disruption of thick filaments, M-lines and dense bodies; disruption is more severe in the muscle of su149 animals. su149 mutant embryos have normal organization of myosin, suggesting that unc-100 is required for the maintenance rather than the initial assembly of myofibrils. Although the genetic map position reported on WormBase for unc-100 on chromosome I is 22.75+/−1.190 cM, we used 3-factor and SNP mapping to place unc-100 between 1.87 and 2.26 cM, which spans 17 overlapping cosmids. After performing transgenic rescue, injecting the
cosmids in sets of three, we were able to conclude that unc-100 lies on one of these three cosmids: ZK524, T28F4 and C26C6. We are currently performing single cosmid rescue experiments to determine which of the three unc-100 lies on. To further accelerate identification of the unc-100 gene sequence, we are conducting whole genome deep sequencing of unc-100 (su149).

**1776/B155**

**Alterations on Striated Muscle Caused by Anabolic Androgenic Steroids Associated with a Selective β-adrenoceptor Blocker.**

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The effects of the association of nandrolone with metoprolol on striated muscle were investigated. Forty male Wistar rats were randomly distributed into four groups: control, treated with nandrolone 10mg/kg Biweekly, treated with metoprolol 1mg/kg/day, and treated with both nandrolone and metoprolol for seven weeks. Left ventricle, soleus, and gastrocnemius sections were cut on a cryostat (5µm), and stained with hematoxylin and eosin, or picrosirius red. Digital images were captured and analyzed by software. Cross-sectional area, diameter, number of myonuclei per fiber, central myonuclei, splitting cells, myonuclear domain, percentage of conjunctive tissue, and serum testosterone were measured. Glucocorticoid (GR) and androgen receptor (AR) were analyzed by immunodetection. An increase was seen in the morphometric parameters analyzed in both cardiac and striated fibers from animal treated with nandrolone. Metoprolol partially restored the cardiac hypertrophy caused by nandrolone without reducing the final percentage of conjunctive tissue. However, the anabolic effect of nandrolone was not reverted by metoprolol on the striated fiber. Nandrolone administration increased serum testosterone levels and up-regulated the expression of AR whereas down-regulated GR expression (P<0.05). We conclude that; (1) the hypertrophic effects caused by nandrolone treatment are accompanied by a higher proportion of conjunctive tissue in cardiac and skeletal muscles, (2) metoprolol administration has a positive effect on the cardiac concentric hypertrophy caused by the steroid hormone, and (3) likely competitive mechanisms of metoprolol in addition to up-regulation of beta-adrenoceptors could have been responsible for the increased fiber size on skeletal muscle after beta1-blocker treatment.

**1777/B156**

**Can the Prostaglandin 15Δ-PGJ2 Influence Skeletal Muscle Regeneration?**

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Many actors classically known to be involved in the inflammatory response can also play an important role in tissue regeneration. Cyclooxygenase-2 (COX-2) is a striking example since there are growing evidences that COX-2-derived prostaglandins could have anti-inflammatory effects. In fact, 15Δ-PGJ2, a PGH2-derived metabolite, has been postulated to be a key actor in the resolution of inflammation and can stimulate fibroblast proliferation. OBJECTIVE: to evaluate if 15Δ-PGJ2 can stimulate L6 myoblast proliferation In Vitro and accelerate skeletal muscle regeneration In Vivo and investigate the mechanism underlying these effects. METHODS: In vitro: L6 myoblasts were submitted to proliferation assays with 15Δ-PGJ2, DP1 agonist and DP1 and DP2 antagonists. In vivo: Female rats were injured with bupivacain in the tibialis anterior muscle, treated with 15Δ-PGJ2 and sacrificed at days 5, 10 and 15. The cross sectional area (CSA) of myofibers and the number of centrally nucleated fibers (CNF) were obtained from muscle sections stained with hematoxyline/eosine while the density of macrophages ED1+ and ED2+ was obtained by immunochemistry. Protein content of myoD and myogenin was evaluated by western blotting. RESULTS: In Vitro cell proliferation was significantly increased in a dose-dependent manner by 15Δ-PGJ2. DP1 and DP2 antagonists inhibited the 15Δ-PGJ2-induced stimulation of myoblast proliferation by 84 ± 17 % and 111 ± 20%, respectively. Surprisingly, DP1
agonist failed to stimulate myoblast proliferation. In Vivo treatment with 15Δ-PGJ2 tended to increase the CSA of injured fibers at day 5 when compared to placebo (776 ± 102 µm² vs 863 ± 67 µm²), but this effect was lost at day 10 and 15. CNF tended to increase with treatment at day 5, 10 and 15. Preliminary results showed that 15Δ-PGJ2 modulated the expression of myoD and myogenin. In summary, 15Δ-PGJ2 can accelerate proliferation of L6 myoblast In Vitro and this effect could be through stimulation of DP1 and DP2 receptors. The tendency to increase CSA and the number of CNF following treatment with 15Δ-PGJ2 suggests that this prostaglandin could shorten inflammation and/or stimulate regeneration. Supported by grants from NSERC and CIHR.

1778/B157
Incomplete Functional Redundancy of Obscurin and Obscurin-like 1 (OBSL1) in Striated Muscle Development.
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Background: Obscurin and OBSL1 are orthologues of the invertebrate Unc-89 gene. In C. elegans, mutation or loss of Unc-89, a giant cytoskeletal protein with both structural and signaling properties, is associated with severe impairment of locomotion and the absence of organized M bands in striated muscle (Waterston et al., 1980). Yet, mice lacking obscurin (Lange et al., 2009) and humans lacking OBSL1 (Hanson et al., 2009) do not demonstrate significant cardiac or skeletal myopathy, suggesting a functional redundancy of the two related proteins. Objectives: In this study, we examined the unique and shared contributions of obscurin and OBSL1 to striated muscle development and myofibril assembly using In Vivo and In Vitro models. Methods: We used morpholino antisense oligonucleotides to reduce expression of obscurin and OBSL1, individually and in combination, in developing zebrafish embryos. Comparison to mammalian models was performed in differentiating C2C12 myoblasts and remodeling adult rat cardiac myocytes. Results: Zebrafish embryos depleted of obscurin a commonly displayed abnormalities of somite segmentation and myofibril alignment that were not noted in embryos lacking OBSL1. Embryos that lacked OBSL1 shared some features with the human OBSL1 deficiency syndrome in that the embryos were shorter with craniofacial abnormalities that were not noted in response to obscurin depletion. Effects of OBSL1 reduction on cardiac structure and function were strain-dependent with cardiac hypoplasia and pericardial edema in those more severely affected. In Vitro models demonstrated that, although obscurin and OBSL1 localized to the M bands of myofibrils, their spatio-temporal distribution suggested both shared and unique functions. Conclusions: Obscurin and OBSL1 have both shared and unique roles in striated muscle development and myofibril assembly. Their ability to compensate for each other appears to be context- and species-dependant. Since OBSL1 lacks the signaling properties of obscurin, it is likely that other cytoskeletal and signaling proteins, outside the obscurin gene family, provide a functional redundancy that may compensate for the loss of obscurin or OBSL1 in some settings.

1779/B158
Myofibril Maturation Is Coordinated with Cardiomyocyte Elongation during Cardiac Chamber Formation.
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Embryonic hearts increase in size and contractile force to cope with rising demand during development. As the heart tube transforms into cardiac chambers, cardiomyocyte size expands to create chamber curvatures. To investigate whether myofibrils mature while cardiomyocytes expand, we used immunofluorescence to examine Z disc dimensions and cell contours in embryonic zebrafish hearts. In wild-type embryos, ventricular outer curvature cardiomyocytes gradually expand in size while simultaneously increasing their myofibril thickness. Thus, it seems that embryonic cardiomyocytes undergo hypertrophic growth similar to that observed in cardiomyocytes in culture, which increase their myofibril content in concert with their size.
expansion. We are currently confirming this coordination in individual cells through time-lapse imaging of a new transgenic line, Tg(cmlc2:α-actinin-gfp). Prior studies have shown that periodic mechanical stretch can trigger hypertrophic growth in cultured cardiomyocytes. To test whether the mechanical impact of blood flow can influence myofibril maturation in vivo, we then examined myofibrils in weak atrium (wea) mutant embryos. In wea mutants, ventricular outer curvature cells fail to expand in size as a consequence of weakened input from atrial blood flow. Despite the small size of wea mutant cardiomyocytes, their myofibrils still significantly thicken. This suggests that the coordination between cell size expansion and myofibril thickening observed in wild-type hearts relies upon proper mechanical input. Ongoing studies explore the molecular mechanisms that link the mechanical stress response to embryonic cardiomyocyte hypertrophic growth, with an emphasis on genes that are differentially regulated in wild-type and wea mutant cardiomyocytes.

1780/B159
The Key to Reorganizing Organelles during Muscle Differentiation: Centrosomal Proteins, Yes, Microtubules, No.
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During muscle differentiation, myotube nuclei become focal points of reorganized centrosomal proteins, organelles, and microtubules. The classic compact Golgi complex (GC) at the centrosome is replaced by a belt of smaller stacks along the myotube nuclear membrane, flanking belts of ER exit sites (ERES) and of the centrosomal proteins pericentrin and γ-tubulin. The microtubule network is reorganized as well. In undifferentiated cells, the microtubule pattern governs the position of organelles. It has not been shown however, that microtubules play a similar role in the formation of the belts during differentiation. We now report that dynamic microtubules are dispensable for the relocation of centrosomal proteins, the GC and ERES, as the formation of belts can proceed in cells chronically treated with the microtubule disrupting drugs nocodazole, taxol or 1-OH. Myogenin expression and pericentrin relocation are necessary but not sufficient for GC and ERES relocation, indicating a hierarchy. A crucial role for centrosomal proteins is further highlighted by the failure of microtubules to reform a normal network after prolonged nocodazole treatment has caused mislocalization of pericentrin. In untreated differentiating cells, we have uncovered intermediate stages in the formation of the belts, indicative of a gradual transition. Partial belts of pericentrin, GC and ERES formed early in differentiation. Pericentrin, GC and ERES eventually wrapped around the entire nucleus like a shell. Lastly, we tracked GFP-tagged EB1 and EB3, and found that newly formed microtubules originate from myotube nuclei. This confirms the role of nuclear envelopes as competent microtubule nucleation sites in untreated myotubes. Previously, microtubule nucleation at nuclei had only been demonstrated in myotubes recovering from microtubule depolymerization. In conclusion, we propose that centrosomal proteins at myotube nuclei pave the way for GC and ERES relocation and steady state microtubule nucleation and that a dynamic microtubule network is dispensable for the relocation of centrosomal proteins, GC and ERES.

1781/B160
Identification of a Binding Partner for UNC-89 (Obscurin) As a New Focal Adhesion Protein, CPNA-1, Required for Muscle Development in C. elegans.
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UNC-89 is a giant modular polypeptide (up to 900,000 Da) required for sarcomeric M-line assembly or maintenance in C. elegans striated muscle. The human homolog of UNC-89 is obscurin. The largest UNC-89 isoform consists of 52 Ig domains, 2 Fn3 domains, a triplet of SH3, DH and PH domains near its N-terminus, and 2 protein kinase domains near its C-terminus. To better understand how UNC-89 functions, we are identifying the binding partners of its many domains. Using Ig domains 1-5 as bait to screen a yeast 2-hybrid library, we identified prey clones representing the gene tag-149. TAG-149 has near its N-terminus a predicted
transmembrane helix, and near its C-terminus, a “copine domain”. The function of the copine domain is speculated to be involved in protein-protein interactions since it is weakly homologous to the extracellular portion of integrins. TAG-149 is an unusual copine family protein in that it has a copine domain but no C2 domains. There are 7 genes encoding copine domain proteins in C. elegans, but only 2 of them are typical in that they possess C2 domains. Therefore, we have changed the name of TAG-149 to “CPNA-1” for “copine domain atypical-1”. Only the copine domain and Ig1-3 are required for the interaction between CPNA-1 and UNC-89. Furthermore, when tested by 2-hybrid, no other region of UNC-89 as bait, showed interaction with CPNA-1 as prey. A GST fusion of Ig1-3 was able to pull out CPNA-1 from a worm lysate. cpna-1 was also identified during a muscle transcriptome-wide RNAi screen to identify genes with the “Pat” phenotype that results from loss of function of genes crucial for muscle development. An intragenic deletion for cpna-1, gk266, displays the typical Pat phenotype of paralysis and arrest at the two-fold stage of embryonic development. The Pat phenotype has been found in loss of function mutants of many components of the muscle focal adhesion structures (M-lines and dense bodies). Affinity purified rabbit antibodies have been generated to CPNA-1, and these antibodies react with a protein of ~130 kDa, the size expected for CPNA-1b. When used in immunofluorescence experiments on whole worms, CPNA-1 was found to localize to both M-lines and dense bodies (Z-disk analogs).

1782/B161
Quantitative Mass Spectrometry-based Protein Profiling of Skeletal Muscle Cell Differentiation In Vitro.
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Skeletal muscle differentiation is a highly dynamic process that contains not only morphological alterations from spindle-like myoblasts to elongated myotubes but also muscle-specific protein synthesis to organize sarcomere structures. The entity of molecules, however, expressed at various stage in the course of muscle cell differentiation is not comprehensively captured. In this study, we have determined proteomic profiles using quantitative mass spectrometry-based analysis (iTRAQ) to assess differences among four distinct muscle culture stages, i.e. (1) growth condition, (2) day 1, (3) day 2, and (4) day 5 after differentiation. Approximately 450 proteins were identified in Triton X-100 soluble fraction of cultured cells. These identified proteins were derived from cytoplasm, mitochondria, nucleus, myofilbril, plasma membrane. In myofilbrils, about 2.0-fold increment in alpha-actin, 2.1-fold increment in troponin T, and 1.3-fold increment in embryonic myosin heavy chain were observed immediately after induction of differentiation. In contrast, as a non-sarcomeric protein, histone family members were down-regulated after differentiation, indicating that the cell cycle was suppressed in differentiated muscle cells. Furthermore, our proteome profiling revealed a sequential isoform expression of myosin heavy chain family. The embryonic type was up-regulated immediately after induction of myogenic differentiation, whereas the perinatal type was not highly up-regulated until day 5. These results indicate that iTRAQ is a simple and useful method for determination of quantitative protein profiles during muscle cell differentiation.

1783/B162
The Role of Stretch-activated Channel on Myogenic Differentiation of Skeletal Myoblast.
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Mechanical stretch regulates the activation of myogenic factors and progression of myoblast differentiation through a complex interplay of signaling pathway. The expression of the typical myogenic markers and myoblast-myotube transition are known to be Ca²⁺-dependent. Data from pharmacological or electrophysiological experiments suggest that the activation of stretch-
activated (SA) channels may play an important role in these processes. However, neither the identity nor the mechanism of activation of SA channels is known. Recently, several members of transient receptor potential (TRP) ion channel family have been implicated to function in mechanosensation and are recognized as the candidate mechanosensitive cation channels. Here, we studied about the role of TRPV2 on myogenic differentiation and migration of a cultured skeletal muscle cells, C2C12. The stretch-induced Ca\textsuperscript{2+} entry was measured by fura2 ratiometry at several stages of C2C12 differentiation from myoblast to mature myotube after switching a growth medium to a differentiation medium. Only just before myoblast-myotube transition, the stretch-induced Ca\textsuperscript{2+} entry was temporarily observed, and completely blocked by the SA channel inhibitors, ruthenium red and gadolinium. Western blot analysis and immunocytochemical staining revealed that the protein expression of TRPV2 was increased in coincident with this temporary emerging Ca\textsuperscript{2+} entry. After this stage of the differentiation, both of the TRPV2 expression and the stretch-induced Ca\textsuperscript{2+} entry were decreased. TRPV2-knocked down C2C12 myoblast with TRPV2-specific morphorino-oligo did not show the stretch-induced Ca\textsuperscript{2+} entry and the transition to myotube. The sarcomeric formation and the expression of myogenic markers, however, still progressed in the TRPV2-knocked down myoblast. These observations strongly suggest that TRPV2 is a responsible ion channel for stretch-induced Ca\textsuperscript{2+} entry needed for the myoblast-myotube transition in C2C12 cells. In contrast, sarcomeric formation and expression of myogenic markers in differentiation of skeletal myoblast are independent of SA channel activity and TRPV2.

**1784/B163**

**Matrix Metalloprotease13 Activity Is Important For Skeletal Muscle Regeneration.**

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Skeletal muscle regeneration requires timely expression of genes for instigating activation, proliferation, and differentiation of satellite cells, as well as genes regulating extracellular matrix remodeling. We have identified a new matrix metalloprotease, MMP13, which is involved in the resolution of muscle damage. To determine the timecourse of MMP expression and activity in regenerating muscle, cardiotoxin (CTX) was injected into the anterior muscle compartment of adult C57 mice, which is a very reproducible method for muscle regeneration. Muscles were harvested 1-11 days post CTX injection and processed for qRT-PCR, zymography and immunoblotting. Mmp9 was elevated early in the repair process, but by day 3 fell to below control muscle values. Mmp2 had transient elevation 1 week after CTX injection. In contrast, Mmp13 expression did not increase until 1 week after CTX injection, and remained high. The changes in MMP expression during regeneration were reflected in zymography of muscle homogenates, and confirmation of MMP13 was achieved by immunoblotting. Next, we examined the source of MMP13 during muscle regeneration by immunohistochemistry. We found that the source of MMP13 was damaged muscle fibers. Finally, we utilized In Vitro myoblast differentiation as a way to determine the steps in which MMP13 might be involved. Pharmacologic inhibition of MMP13 caused a reduction of myoblast fusion and myotube formation compared to control cultures. These results support that MMP13 contributes to muscle regeneration by promoting myoblast fusion to sites of damage, which may accelerate muscle repair.

**Kinesin (1785 – 1800)**

**1785/B164**

**A 'Catalytic Engine' That Accounts For Kinesin Motility and Directionality.**

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We have characterized the microtubule-attached conformations and hydrolysis cycles of two oppositely-directed kinesin motors, conventional kinesin and ncd, by cryo-electron microscopy, imaging at a resolution (8-9Å) sufficiently high to discriminate between different conformations of
small, key secondary structure elements. Consequently, we were able to assemble composite atomic models of these motors' pre- and post-power stroke states while attached to the microtubule, by utilizing the large ensemble of available motor protein crystal structures as a conformational library. These models describe an elegant mechanism accounting for microtubule-activated force generation and hydrolysis. Microtubules drive the reaction by triggering a disorder-to-order transition at the N terminus of the motors' 'switch II helix' element (forming a 'switch II helix extension'), which subsequently stabilizes both (1) the pre-power stroke conformation prior to ATP binding, and (2) the catalytically active post-power stroke conformation following ATP binding. Key to the mechanism is the appearance, upon ATP binding, of a 'closed' arrangement of the nucleotide-sensing 'switch' loops, previously visualized only in crystal structures of kinesin's ancestral relative, myosin; this closure is stabilized by a constellation of complementary interactions between the switch loops and the switch II helix extension, explaining why microtubule attachment (and accompanying nucleation of the extension) is required for kinesin activation. In ncd, we also detected the likely presence of a structural homolog of conventional kinesin's force-delivering 'neck linker', which we propose to call the 'pseudo linker'. We subsequently identified a probable mechanism by which the post-power stroke conformation of the pseudo linker would stabilize ncd's putative force amplifier, known as the 'neck', in a novel orientation directed towards the minus end of the microtubule, representing the end state of ncd's power stroke. The conformations we have inferred thus account for the principle features of filament activation and direction reversal in the kinesin superfamily, and also have certain implications for the motility mechanism of myosin.

1786/B165
Stalk Rotation and Steps by a Kinesin-14 Motor.
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The nonprocessive kinesin-14, Ncd, releases from the microtubule after each step it takes, rather than taking many successive steps, like kinesin-1. The basis of Ncd steps is essential to understand how the motor works, but is currently unknown. Ncd steps are fundamentally different from kinesin-1 in that they occur upon binding by a single head of the dimeric motor to a microtubule, rather than by alternate head binding; they are also frequently off-axis rather than parallel to the microtubule axis and rotation of the stalk is thought to drive steps by the motor, instead of docking/undocking of the neck linker. Here we show by mutant analysis and FRET between the end of the stalk and microtubule that rotation of the Ncd stalk occurs in two phases - the stalk initiates movement when the motor binds to a microtubule and releases ADP, but the end of the stalk remains ~9 or more nm from the microtubule. The stalk then rotates within ~6 nm of the microtubule surface when the motor binds ATP. Assuming an initial vertical position, the stalk rotates ~70 degrees, consistent with the ~75-degree rotation observed in a crystal structure. This would produce a step of ~19 nm for full-length Ncd with a stalk of ~20.2 nm. An initial 46 degree tilt of the stalk towards the plus end, measured from a cryoEM map, would increase the step size to ~34 nm, four times larger than the 8-nm step of kinesin-1. The ability to lever an attached load means that Ncd steps are not constrained by motor binding sites along microtubules like kinesin-1. Instead, step size is determined by the stalk length and rotation angle, including the initial tilt towards the plus (or minus) end and off-axis bias. The step size may be stochastic, as observed for single Ncd molecules in laser trap assays, and constrained by motor cooperativity, explaining the slower velocity of Ncd than kinesin-1. Interactions with other rigid, rod-like proteins in the spindle could influence the step size by altering the rotation angle of the stalk, including its initial tilt, or change its effective length. This may play an important, previously unrecognized role in Ncd spindle function and have mechanistic implications for the function of other motors.

1787/B166
Manipulation of Neuronal Vesicular Transport and the Mechanism of KIF5-Driven Polarized Axonal Transport.
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In pursuit of the mechanism of polarized transport in neurons, we previously showed that truncated kinesin-1 (conventional kinesin, KIF5) selectively accumulates at the tips of axons. However, it remains unknown whether KIF5 pulls vesicles specifically to axons in vivo, and, if so, what kind of cue KIF5 recognizes in axonal microtubules. Using rapamycin-dependent heterodimerization method, we showed that simple binding of KIF5 causes polarized axonal transport of axonal, dendrite, and ER-to-Golgi vesicles, indicating the key role of KIF5 in polarized axonal vesicular transport. Concerning the cue in microtubules, we found a novel molecular difference between axonal and somatodendritic microtubules. Pharmacological treatment, In Vitro motor binding assay, and antibody-inhibition assay suggested it was crucial for polarized accumulation of truncated KIF5 at the axonal tip. Our study suggests that there is a novel molecular difference between axonal and dendritic microtubules, and KIF5 recognizes it to achieve polarized axonal transport.

1788/B167
The Extended Neck Linker of Kinesin-2 Diminishes Its Processivity.
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Kinesin-2 motors, which are involved in intraflagellar transport as well as cargo transport along cytoplasmic microtubules, possess a neck-linker domain three amino acids longer than the corresponding domain in Kinesin-1. The neck-linker domain is a key structural element in force generation by kinesins, and because any mechanical communication between the two head domains must be transmitted through the neck linker, its mechanical properties also play an important role in chemomechanical coupling. Using single-molecule bead experiments, we found that Kinesin-2 is four-fold less processive than Kinesin-1. To test the mechanistic basis of this difference, we inserted the last three amino acids of the Kinesin-2 neck linker into Kinesin-1 and found that the Kinesin-1 processivity fell five-fold with only a small decrease in the motor velocity. Smaller neck-linker insertions into Kinesin-1 also diminished motor processivity and surprisingly, shortening the Kinesin-2 neck linker enhanced its processivity. These results suggest that the mechanical properties of the neck linker play an important role in head-head coordination in kinesins, and that there is an optimal length that is sufficiently long to enable the tethered head to diffuse to its next binding site, but short enough to provide a stiff connection between the two head domains when bound to the microtubule. Molecular dynamics simulations of the Kinesin-1 and Kinesin-2 neck linkers provide estimates for their mechanical properties and kinetic simulations of the kinesin hydrolysis cycle suggest that extended neck linkers lead to uncoupling of the front head gating mechanism. The correlation between longer neck linkers and reduced processivity may be a general paradigm that relates structure to cellular function across the kinesin superfamily.

1789/B168
Novel Kinesin Superfamily Protein 26A (KIF26A): A Key Regulator of GDNF-Ret Signaling in Enteric Neuronal Development.
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The kinesin superfamily proteins (KIFs) play multiple roles in cellular function. In the present study, we identified KIF26A, a new member of the murine KIFs. Due to a non-conserved consensus motif for ATP hydrolysis, KIF26A is rather atypical and lacks ATPase activity. Kif26a knockout (kif26a-/-) mice was analyzed to understand the functional role of KIF26A in vivo. Kif26a-/- mice developed a megacolon with enteric nerve hyperplasia. In kif26a-/- enteric neurons, the glial cell line-derived neurotrophic factor (GDNF)-rearranged during transfection (Ret) signaling exhibited hypersensitivity. Results showed that KIF26A suppressed GDNF-Ret
signaling by binding with the SH2 domain of Grb2, an essential component of GDNF-Ret signaling. KIF26A inhibited GDNF-induced formation of the SHC-Grb2 complex and suppressed activation of the GDNF-Ret cascade in the enteric nervous system. We, therefore, propose that KIF26A plays a key role in enteric nervous system development by regulating GDNF-Ret signaling.

1790/B169

**Proposed Model for Kinesin Kar3Cik1 Interpolar MT-MT Crosslinking Function during Mitosis.**

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Kar3Cik1 is a S. cerevisae Kinesin-14 motor protein that promotes microtubule (MT) shortening during karyogamy yet acts to crosslink interpolar MTs (ipMTs) during anaphase (3,4). The Kar3 head contains both an ATP and MT binding site, yet there is no nucleotide binding site in Cik1(1). Presteady-state and steady state experiments have been pursued to define the mechanism by which Kar3Cik1 performs its mitotic function to crosslink and stabilize anti-parallel ipMTs. We have developed an approach to begin our experiments with a homogenous population in which the Cik1 head binds to the MT first followed by the Kar3 motor domain (Kar3MD). The MT association kinetics at 4.9 μM-1s-1 are fast followed by Kar3MD association and rapid ADP release at 109 s-1. ATP binding to the Kar3MD is also a fast step at 2.1 μM-1s-1 with koff = 16.6 s-1. ATP-promoted dissociation of the MT-Kar3Cik1 complex occurs at 11.5 s-1 with the motor detachment occurring after ATP hydrolysis. However, the rate-limiting step for steady-state ATP turnover at 5 s-1 has not yet been identified. These initial results suggest a model in which Kar3Cik1 interacts with the MT through an alternating cycle of Cik1 binding followed by Kar3MD binding. Because Cik1 does not have a nucleotide binding site, we propose that head-head communication is mediated by a strain-dependent mechanism.

1791/B170

**Five Kinesins-like Proteins and Intracellular Transport in Posterior Silkgland Cells of Silkworm.**

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Kinesins are microtubule-based motors widely participating in various intracellular transports, the mechanisms of which, however, remain elusive. Here, we chose to use specialized posterior silkgland (PSG) of silkworm, which possesses an extensive endomembrane system for highly efficient secretion of substantial fibroin, as a novel model for this study. To investigate kinesin-driven intracellular transport in PSG cells, we cloned five novel silkworm kinesin-like proteins (KLPs), BmKLPa, BmKLPb, BmKLPc, BmNcd, and BmKHC, which belong to Kinesin-13, Kinesin-7, Kinesin-6, Kinesin-14A, and Kinesin-1 subfamilies, respectively. Using relative real-time PCR and Western blotting, we determined tissue-specific and silkgland developmental expression patterns of these five KLPs. Immunofluorescence experiments in BmN cells showed that BmKLPs colocализed with microtubules, except for BmKLPb, while transfection studies in PSG cells showed a colocализation of BmKHC and BmNcd with microtubules. By pull-down assays, LC-MS/MS and Western blotting analysis, we surprisingly identified BmKHC as a motor protein that transported many potential cargoes in PSG, including fibroin granules and exuperantia-associated ribonucleoprotein (RNP) complexes. Moreover, BmKLPa overexpression led to microtubule depolymerization, suggesting that BmKLPa processed microtubule-depolymerizing activity. Based on these results, PSG could serve as a potential model to reveal the mechanism of kinesin-driven intracellular transport in secretory tissues.

1792/B171

**Evidence to Suggest That Weak Coiled-Coil Association between the Kinesin-2 Motor Subunits Is Functionally Important In Vivo.**
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Kinesin-2 is a heterotrimer of two dissimilar motor subunits and an accessory protein found in all organisms from Drosophila to humans. The motor subunits are known to associate through coiled-coil interactions between two dissimilar stalk domains at the middle. However, the mechanism and utility of such interactions on the motor functions were unclear. Our study is aimed at understanding the structural basis of association between the motor subunits KLP64D and KLP68D of Drosophila. Systematic analysis of truncated fragments of recombinant stalks expressed in bacteria showed that the motor subunits associate with each other through incomplete coiled coil interaction between the C-terminal one-third portions. The recombinant KLP64D stalk is unstable. However, it formed a stable but weak coiled-coil with the KLP68D stalk when co-expressed in bacteria. Although the N-terminal portions of the KLP64D and KLP68D stalk fragments didn’t associate with each other, a conserved Gly to Cys change at the N-terminal region of KLP68D stalk destabilized the entire assembly, indicating that the long range interaction along the stalk plays a crucial role in the heterodimerization. In contrast, another missense mutation (E551K) in the C-terminal part of the KLP64D stalk, which was known to cause severe lethality in Drosophila, enhanced the coiled-coil association with KLP68D, which was reversed by additional missense mutations along the stalks. Altogether, these results indicate that weak and partial coiled-coil association between the stalks of kinesin-2 motor subunits is crucial for maintaining its function in vivo.

Mechanism of Force Generation by Mitotic Kinesin-5 Molecular Motors.
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The mitotic spindle is essential for faithful cell division. It is built from microtubules and is orchestrated by many proteins, including members of the kinesin superfamily. Kinesin-5 motors are essential for mitosis in many organisms and are involved in formation and maintenance of spindle bipolarity. Kinesin-5s share some properties with other kinesins including the ability to move - albeit slowly - towards the plus ends of microtubules. However, kinesin-5s have a number of unique properties and are also of interest for cancer treatment because kinesin-5-specific small molecule inhibitors have been identified and are in clinical trials. Outstanding mechanistic questions about kinesin-5 motors relate to their interaction with microtubules. We set out to understand this interaction using cryo-electron microscopy and image processing. Cryo-electron microscopy is uniquely suited to this goal since microtubules are too large and heterogeneous to be studied by other structural techniques. Using the motor domain from KLP61F (the Drosophila kinesin-5), we imaged microtubules bound by the motor in an ATP-like state and calculated the structure of the complex at 9Å resolution. At this resolution, we are able to see the density associated with most α-helices in both the motor and the microtubule, and to visualise the motor in a tight-binding conformation. The docked tubulin structure shows an excellent fit to our density, but available kinesin-5 crystal structures do not match the conformation of the motor in our reconstruction, indicating that microtubule binding induces conformational changes in the kinesin-5 motor. Our structure also provides insight into the mechanisms by which anticancer drugs elicit their therapeutic effect. Thus, calculation of kinesin-microtubule structures are essential for revealing the precise mechanism by which motors use energy from ATP and microtubule binding to generate force and emphasizes that the kinesin-microtubule complex forms a force-transducing holoenzyme.

Kinesin’s Light Chains Inhibit the Head- and Microtubule-binding Activity of its Tail.
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The kinesin-1 holoenzyme comprises two heavy chains and two light chains (KLCs). The goal of this study was to dissect the function of KLCs in kinesin-1 regulation, as previous work in this area has yielded conflicting results. We used biochemical methods with purified proteins In Vitro to describe a novel function of the KLCs - the ability to regulate the head- and microtubule-binding activity of the heavy chain tail. Kinesin-1 ATPase activity is inhibited through a direct head-tail interaction, and we show by fluorescence anisotropy that KLCs reduce the affinity of this interaction ten-fold. We also show in pelleting assays that KLCs block tail-microtubule binding, which is mediated largely by electrostatic interactions with the acidic tubulin C-terminus. Inhibition is pH-dependent, with high inhibition physiologically (pH 7.4) and low inhibition under acidic conditions (pH 6.6). Inhibition of tail binding to heads and microtubules does not require the KLC cargo-binding domains, and is likely due to the negatively-charged character of the KLCs. Thus, we demonstrate that KLCs can act as a general regulator of kinesin-1 by modulating the behavior of its tail. Our data support a model wherein KLCs promote activation of kinesin-1 for cargo transport, while simultaneously allowing the motor to bypass transport-incompetent (tail bound to microtubules instead of cargo) states in its motile phase. Inhibition of tail-microtubule binding may also influence kinesin-1’s role in microtubule sliding and cross-linking, with particular regard to cytoplasmic streaming processes.

1795/B174
The Role of the CYK-4 GAP Subunit in Centralspindlin-Microtubule Interaction.
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Centralspindlin is a key factor in organizing the spindle midzone during cytokinesis. It is a tetrameric protein complex composed of a kinesin-like protein (ZEN-4 in *Caenorhabditis elegans* and MKLP1/CHO1 in mammals) and a GTPase activating protein (GAP) for Rho-family GTPases (CYK-4 in *C. elegans* and MgcRacGAP in mammals). At the midzone it is known to have two integral roles. First, it recruits various proteins needed for efficient cell division such as ECT-2 and FIP3 through direct interaction with the CYK-4 subunit. The second function of centralspindlin is to bundle microtubules (MTs) helping to shape anaphase spindle structure and to form the midbody which is important for abscission. It has previously been shown that effective MT bundling activity requires both ZEN-4 and the N-terminal domain of CYK-4, which binds the neck region of ZEN-4. However, it is currently unknown how this promotes bundling of MTs. To address this we performed In Vitro experiments with the minimum ZEN-4 binding domain of CYK-4 (CYK-4N) and observed the effect on the interaction between dimeric ZEN-4 constructs and microtubules. In surface-gliding assays, where ZEN-4 is immobilized on a glass surface, CYK-4N caused significant reduction of the velocity of microtubule translocation. When microtubules were immobilized on the surface, ZEN-4 in solution labeled the microtubules and accumulated to their plus-ends in both the presence and absence of CYK-4N. If additional free microtubules were introduced, they were bundled with the immobilized microtubules in a ZEN-4 dependent manner. Interestingly, CYK-4N increased the efficiency of accumulation of ZEN-4 to the bundled region of microtubules. Together, these results indicate that CYK-4 binding regulates the activity of ZEN-4 kinesin to interact with microtubules.

1796/B175
Characterization of the Novel Rice Kinesin O12 Which Has CH Domain.
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Recently, the genome sequence of the rice *japonica* has been completed. From the genomic analysis, it is predicted that rice *japonica* has at least 38 kinesin-like (kinesin-related) proteins whereas some kinesins do not classified into known subfamilies. Therefore, we expressed some rice kinesins. In analysis on the genome, the rice kinesin O12 is one of them was presumed that it is belongs to kinesin family-14. In addition, the rice kinesin O12 has calponin homology domain (CH domain) in N-terminal. CH domain is known in the actin-binding domain. To date the KCH
kinesins (Kinesins with CH domain) have been found only in higher plants. In this study, we have clarified the interaction of the kinesin O12 and actin using biochemical methods. ATPase activities in the presence and absence of actin were analyzed and compared with those of other kinesins. ATPase activities of the O12 CH-MD (CH domain and motor domain) were significantly reduced in the presence of actin. ATPase activity of O12 MD also decreased in the presence of actin. Apparent Ki of O12 CH-MD and O12 MD for actin were 5 \( \mu \text{M} \) and 20 \( \mu \text{M} \), respectively. on the other hand, ATPase activity of conventional kinesin MD was not affected by actin. The results of co-precipitation of kinesin with actin also suggested that the O12 CH-MD significantly interact with actin. Localization of O12 in the cell has been studied. O12-tail fused with GFP was expressed in the onion cell and the fluorescence of GFP was seen in the shape of filaments corresponding to actin. Therefore, it was suggested that O12 interact with actin in the living cell.

1797/B176
Selective Translocation of Kinesin Motor Domains in Cultured Hippocampal Neurons.
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Polarized transport is crucial for neuronal development and function. It is thought that some kinesins recognize biochemical differences between axonal and dendritic microtubules and selectively deliver their cargoes to these domains. In support of this possibility, a truncated KIF5C lacking the autoinhibitory domain expressed in cultured neurons accumulates selectively at the tips of axons, but not dendrites; truncated KIF1A accumulates at the tips of both axons and dendrites (Nakata and Hirokawa, 2003; Jacobson et al., 2006). We prepared truncation constructs consisting of the motor, neck, and putative dimerization domains of members of the Kinesin-1, -2, -3, and -4 families and evaluated their ability to accumulate at neurite tips when expressed in cultured hippocampal neurons. Most of the constructs we examined translocate to neurite tips. Truncation constructs of the heterodimeric Kinesin-2s also accumulated at neurite tips when co-expressed with appropriate partners (KIF3A/3B and KIF3A/3C). We were unable to produce truncated constructs of the Kinesin-3 family members KIF13A or 13B that accumulated at neurite tips, but chimeric constructs that included the KIF5C neck-coil and dimerization domains did accumulate. These results indicate that truncation constructs of most kinesins that are dimerization-competent translocate spontaneously when expressed in cultured neurons. Kinesins from the same family did not necessarily share the same patterns of accumulation. for example, KIF3A/3C dimers accumulated in both axon and dendrite, whereas KIF17 and KIF3A/3B dimers accumulated selectively in the axon. Most kinesins exhibited the same pattern of accumulation at all stages of development, but there were exceptions. for example, KIF21A only accumulated at neurite tips in mature neurons, suggesting that developmental modifications to microtubules regulate kinesin activity. By expressing chimeric constructs of related kinesins that exhibit different spatial or temporal patterns of accumulation, it should be possible to identify those sites within the motor domain and elsewhere within the protein that govern the selectivity of kinesin translocation in living neurons.

1798/B177
GSK3/Shaggy Negatively Regulates Bidirectional Axonal Transport of the Amyloid Precursor Protein.
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Axonal transport is essential for neuronal function and survival, and misregulation of transport may be an early event in Alzheimer’s disease (AD). The serine-threonine kinase GSK3, which is likely a central player in AD, has been previously proposed as a regulator of anterograde axonal transport mediated by kinesin-1. The objective of the current study is to determine whether endogenous GSK3 plays a required role in axonal transport, and in particular whether it regulates the movement of the Amyloid Precursor Protein (APP), a key protein in AD that moves bidirectionally in axons as a cargo of kinesin-1 and dynein. Using a loss-of-function approach in Drosophila melanogaster, we find that endogenous GSK3 (Shaggy/Sgg) is required for axonal
transport. Genetic interactions between sgg and kinesin heavy chain support the model that GSK3 acts as a negative regulator of kinesin-1. However, using a live imaging assay in single Drosophila axons, we find that endogenous GSK3 negatively regulates both anterograde and retrograde APP transport. As GSK3 is a known microtubule (MT) regulator, one possibility is that GSK3 regulates all axonal transport indirectly by altering MT stability. We find that loss of GSK3 alters MT acetylation, but that acetylation changes do not account for the transport defects caused by GSK3 reduction. Growing evidence suggests that kinesin-1 and dynein may coordinate each other’s activity, in which case GSK3 could regulate dynein indirectly by targeting kinesin-1. We are currently examining the transport of a bidirectional kinesin-3/dynein cargo to determine whether a. transport by other kinesins is regulated by GSK3 and b. whether GSK3 targets dynein indirectly via kinesin-1 regulation. Conclusions: We have demonstrated that GSK3 negatively regulates bidirectional transport of APP in axons. This is the first demonstration that dynein-mediated movement is regulated by GSK3. In addition, these findings may have important implications for Alzheimer’s disease, in which both GSK3 and APP play central roles.

1799/B178
Multiple Motor Activities Support Cell Polarity and Fusion of Differentiating Muscle Cells.
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Upon differentiation, myoblasts leave the cell cycle, change their morphology to a bipolar spindle shape before they fuse predominantly at their tips to form a muscle fiber. Microtubules are crucial in this process, forming a parallel array with the plus ends extending towards the tips of elongating cells. Maintenance of cell polarity and acquisition of fusion competence is therefore likely to involve kinesin-mediated transport of important, as yet unknown factors towards the cell tips. Here we show that multiple kinesin motors are required for cell elongation and/or fusion during myogenic differentiation. In an initial kinesin-wide screen we identified 16 kinesins that were either upregulated or strongly expressed throughout differentiation. Short interfering hairpin RNA constructs targeting these candidates were used for functional analysis in differentiating C2C12 cells. 13 kinesins were required for efficient cell-cell fusion. 8 of these were also needed for proper cell elongation and showed various cell morphological defects when depleted. Besides two kinesins, Kif2a and Eg5, that are expected to affect microtubule organization, we identified 11 supposed organelle transporters. This suggests that directed transport along microtubules is indeed crucial for cell polarity and the acquisition of fusion competence in differentiating muscle cells. The identification of specific cargo molecules for these motors is currently under way. Thereby, we expect to isolate novel factors that are important for the establishment and/or maintenance of cell polarity and hope to understand how their correct spatial distribution supports changes in cell morphology.

1800/B179
Microtubule Depolymerization by the Kinesin-8 Motor Kip3p: A Mathematical Model.
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The kinesin-8 family includes motors which promote microtubule (MT) depolymerization, and can show plus-ended directed motility on MTs. We have developed a simple model that incorporates both directional motion and MT plus-end destabilization. We focused on length-verses-time traces for stabilized MTs in the presence of purified Kip3p, a member of this family. We were able to quantitatively reproduce key features, particularly the length-dependent depolymerization. We propose that Kip3p depolymerizes processively; each motor results in the removal of more than one tubulin dimer from a stabilized MT. In addition, we determine the parameter regime for length-dependent depolymerization, and showed that MT length fluctuations are related to the motor depolymerization processivity.
Microtubule Dynamics and Assembly II (1801 -1818)

1801/B180
Midzone Alignment and Cleavage Plane Setup in Mammalian Cytokinesis.
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During cytokinesis, to correctly position a contractile ring on the cortex relies on stable signals from microtubules. These signals from midzones then further guide furrow ingression and completion. Different to mitosis, microtubules in cytokinesis are less dynamic, and form midzones in an antiparallel manner. Proteins involved in signaling the cortex, such as PRC1, Centralspindlin, and Rho-GEF Ect2, are all localized on the interdigitated region of midzones. Thus, it is critical to properly align overlapping midzones in cytokinesis, to assure midzone proteins localized coherently to signal a narrow furrow region on the broad cortex. Indeed, failure in midzone alignment impedes the furrow ingression and completion. After cytokinesis onset, midzone proteins initially diffuse along midzones but gradually concentrate to the center of midzones, and finally localize on the same plane in Z-axis. This plane then becomes the cleavage plane for cytokinesis. So far, it is still mostly unknown how midzone proteins and overlapping regions on individual microtubule bundles register each other and be precisely aligned on the cleavage plane without any contact and direct interactions among individual microtubule bundles. Here we showed this coherent positioning mechanism is very robust and independent to actin and myosin systems. It is early initiated, about at the same time chromosome segregations start. In addition, well aligned chromosomes in metaphase equator seem to enhance the midzone alignment process. Midzone misalignment can only happen when individual microtubule bundles are physically isolated. It suggests a possible diffusible signal is involved in the communications among individual microtubule bundles, and also between midzones and the cortex.

1802/B181
Inhibition of HDAC6 Activity Increases the Acetylation Level of Microtubules and Suppresses Microtubule Dynamics in MCF-7 Cells.
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Histone deacetylase 6 (HDAC6) is a tubulin specific deacetylase which removes acetyl group from lysine 40 of α-tubulin. Trichostatin a (TSA) is known to inhibit the activity of histone deacetylases. We examined the effect of tubulin acetylation on microtubule dynamics using TSA and small interfering RNA targeting HDAC6. TSA was found to inhibit the proliferation of MCF-7 cells with an IC₅₀ of 54 nM. Western blotting and immunofluorescence microscopic analysis showed that microtubules of TSA-treated MCF-7 cells were more acetylated as compared to that of the control cells. Microtubules of TSA-treated MCF-7 cells were also more resistant than control cells towards cold depolymerization. TSA treated cells showed resistance against microtubule depolymerizing agents such as nocodazole and colchicine indicating their increased stability. TSA was also found to suppress the dynamic instability of individual microtubules in MCF-7 cells. for example, 30 nM TSA reduced the shortening rate of microtubules by 30 % (21.7±6 in control to 15±4 µm/min). In addition, it increased the percentage of time spent by microtubules in pause state from 33% to 62%. TSA (30 nM) reduced the dynamicity (dimer exchange from the microtubule ends) of microtubules by 63%. In addition, the combination of TSA with taxol (a microtubule stabilizing agent) had a stronger inhibitory effect on cell proliferation suggesting that TSA had stabilizing effects on cellular microtubules. However, TSA did not influence the assembly of purified tubulin In Vitro suggesting that it does not directly stabilize microtubules. To further discern the effect of HDAC6 on microtubule acetylation, stability and dynamics, MCF-7 cells were transfected with HDAC6 siRNA. There was a 3 fold reduction in HDAC6 level after siRNA treatment. We observed a 37% increase in acetylation levels of tubulin following HDAC6 depletion by siRNA. Similar to the TSA treated cells, the microtubules of the cells treated with siRNA for HDAC6, showed an enhanced stability against cold induced
depolymerization. This suggested that HDAC6 has a role in the microtubule dynamics and stability. JA and RM contributed equally to the work.

1803/B182
San Regulates Microtubule Dynamics by Acetylating β-tubulin.
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Microtubule, composed of αβ tubulin heterodimers, is critical for various cellular processes. Different post-translational modifications, including acetylation, diversify microtubules to fulfill different functions. Here we show that San, an acetyltransferase required for sister chromatids cohesion during mitosis, acetylates β-tubulin in vitro. HeLa cells depleted of San reassemble microtubules faster than mock cells after cold treatment, suggesting that San negatively regulates microtubule dynamics in vivo. To determine whether San acts through acetylating β-tubulin, the acetylation site on β-tubulin was mapped by both mass spectrometry and site-directed mutagenesis. β-tubulin mutants mimicking the acetylated form are slower to be incorporated during microtubule reassembly, supporting the idea that San-mediated acetylation on β-tubulin interferes with tubulin polymerization. Taken together, our data suggest that San negatively regulates microtubule dynamics by acetylating β-tubulin.

1804/B183
Augmin Is Required for de Novo Microtubule Generation During Central Spindle Formation.
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Central spindle, which is formed between 2 masses of segregated chromosomes during anaphase, provides a scaffold for signaling molecules involved in proper progression and completion of cytokinesis. The central spindle consists of anti-parallel bundles of microtubules whose minus ends are located away from the centrosomes; however, sources of these microtubules are largely unknown. We investigated the role of de novo microtubule generation in the formation of the central spindle in HeLa cells. Using a microtubule-disassembly/regrowth assay, we observed microtubules regrowing both from the centrosomes and the proximal region of the chromosome away from the centrosomes in anaphase cells. Continuous observation revealed that the microtubules that regenerated from the latter region grew into the inter-chromosomal space and finally formed the central spindle. Most cells were able to complete cytokinesis after microtubule regrowth, suggesting that a fully functional central spindle can be formed solely via de novo microtubule generation during anaphase. A microtubule nucleator, gamma-tubulin, was found to be localized on the central spindle and specifically concentrated at its ends near the separated chromosomes, where microtubule regeneration was observed in the regrowth assay. RNAi experiments revealed that this gamma-tubulin localization depends on augmin, a protein complex that is also required for the localization of gamma-tubulin within the pre-anaphase spindle. In the regrowth assay using augmin-depleted anaphase cells, microtubule regeneration in the inter-chromosomal region was specifically attenuated, while that in the centrosomes remained intact. This resulted in the failure of central spindle formation and completion of cytokinesis. When anaphase cells with a monoastral spindle were created by depletion of Plk4, the central spindle was formed in both hemispheres, suggesting that the centrosome is not required for central spindle formation. These results suggest that de novo microtubule generation during anaphase, which is augmin-dependent but centrosome-independent, serves as a source of microtubules for central spindle formation and contributes to the spatiotemporal regulation of cytokinesis.

1805/B184
Bi-directional Regulation between Microtubules and the Extracellular Matrix in Endothelial Cell Branching Morphogenesis.
Extra-cellular Matrix (ECM) dimensionality and stiffness regulate endothelial cell branching morphogenesis via mechanosensitive cell adhesion receptors that elicit bidirectional signals to and from the actomyosin cytoskeleton. Stiff ECMs promote Myosin II activity that inhibits cell branching, while compliant ECMs reduce Myosin II activity and promote branching. The microtubule (MT) cytoskeleton controls cell morphology through its structural and regulatory interactions with actomyosin. However, the role of MTs in cellular responses to ECM properties, and the effects of ECM properties on MT organization and dynamics are unknown. To explore the role of MTs in cell responses to ECM dimensionality and compliance, we analyzed MT and actin organization in HUVEC cells on two different stiffnesses of 2D and 3D ECMs. This showed that stiffer substrates enhanced MT presence in cell branches. Depolymerization of MTs with nocodazole induced actin stress fibers and inhibited cell branching independent of substrate stiffness or dimensionality, showing that MT depolymerization-induced activation of contractility is not regulated by physical properties of ECM. In contrast, stabilization of MTs with taxol inhibited MT presence in branches. To determine how ECM stiffness and dimensionality affect MTs, we analyzed MT dynamics by tracking fluorescently-tagged MT plus ends. This revealed that on soft substrates, MT growth rate was independent of ECM dimensionality, while on stiff substrates, MT growth was faster in 3D compared to 2D ECMs. In 2D, MT growth rate was independent of stiffness, while in 3D, stiff ECM promoted faster MT growth than soft ECM. Thus, the fastest MT growth occurred in stiff 3D ECMs, and the slowest occurred in 2D ECMs, independent of stiffness. Inhibition of Myosin II with blebbistatin increased MT growth rate under all conditions of ECM dimensionality and stiffness, indicating that Myosin II regulates MT growth independent of ECM physical properties. Furthermore, blebbistatin did not abrogate the effects of ECM stiffness on MT growth. Thus, we find a bidirectional regulation scheme where MTs mediate cellular responses to ECM, and ECM regulates MTs by both contractility-dependent and independent mechanisms.

**1806/B185**

**MCAK Activity Controls Interphase Microtubule Dynamics and Directed Cell Migration.**

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Directional cell migration is initiated through extracellular stimuli that coordinate changes in the cytoskeleton to establish a polarized cellular morphology. Cell polarity can be achieved through regional regulation of microtubule (MT) dynamics, including MT growth toward the leading edge and MT shortening in the cell rear. Mitotic Centromere Associated Kinesin (MCAK) is a MT depolymerase that is down-regulated in mitosis by Aurora kinase phosphorylation. While its mitotic functions have been well-characterized, whether MCAK regulates MT dynamics during cell migration is not known. We hypothesize that MCAK is down-regulated locally via a Rac1/Pak1/Aurora-A kinase signaling pathway to establish preferential MT growth toward the leading edge and to promote MT shortening within the cell rear. To test this hypothesis, we performed time-lapse imaging of fluorescently tagged EB3 as a marker of MT plus-end growth in HUVEC cells and analyzed MT dynamics and cell behavior under different manipulations of the proposed signaling cascade. We find that MCAK knockdown (KD) produces expected effects on the MT cytoskeleton, including increased levels of tubulin polymer and decreased MT catastrophe frequency. MCAK-KD cells show a reduction in MT polymerization speeds and exhibit a mal-oriented MT array, as well as a statistically significant reduction in cell migration velocity, distance, and directionality, indicating a defect in cell migration and/or polarization. These effects are rescued through expression of exogenous wild-type-MCAK, but not by expression of either an inactive (ATPase-dead) MCAK mutant or an MCAK mutant that is incapable of phospho-regulation by Aurora kinases. Immunolabeling of cells expressing either constitutively active-Rac1 or constitutively active-Pak1 suggests that Rac1 and Pak1 activities correlate with increased
Aurora-A activity, as assayed with a phospho-specific antibody, and also correlate with decreased levels of MCAK expression. These data suggest that interphase regulation of MCAK is achieved downstream of a Rac1/Pak1/Aurora-A signaling pathway in order to locally coordinate MCAK-mediated MT depolymerization as a method to ensure proper cell polarization and motility.

1807/B186

**Amplification of Ionic Currents through the Microtubule Lumen by C-Terminal Tails.**

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It has been suggested that microtubules and other cytoskeletal filaments may act as electrical transmission lines. An electrical circuit model of the microtubule is constructed incorporating features of its cylindrical structure with nanopores in its walls and is used to test whether such features might contribute to the proposed role of the microtubule as a mediator in intracellular signaling. Based on the results of Brownian dynamics simulations, the nanopores were found to have asymmetric inner and outer conductances, manifested as nonlinear IV curves. Our simulations indicate that a combination of this asymmetry and an internal voltage source arising from the motion of the C-terminal tails can allow a current to be pumped across the microtubule wall and propagate down the microtubule through the lumen. This current is demonstrated to add directly to the longitudinal current resulting from an external voltage source, and could be significant in amplifying low-intensity endogenous currents within the cellular environment.

1808/B187

**Atypical PKC Dramatically Alters Microtubule Organization through Src Activation.**

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The atypical protein kinase C (aPKC) subfamily of PKC plays a critical role in controlling cell growth and survival as well as cell polarity by associating with the cytoskeleton. The aPKC pseudosubstrate (aPKC-PS) domain is different from the other PKC family members and a synthetic peptide corresponding to the PKC pseudosubstrate domain can be used to determine substrate specificity and to characterize the physiological response of the phosphorylated substrate(s). Moreover, the aPKC-PS sequence contributes to aPKC binding to microtubules (MTs). Although aPKC binds directly to MTs, sparse information is available regarding the role of this signaling molecule in MT dynamics. In this study, we found that introduction of the aPKC-PS domain into HeLa cells caused a striking change in MT organization that resulted in the appearance of MT-rich membrane protrusions. Dynamic MTs are required for protrusion formation because treatment with taxol or nocodazole prevented the phenotype. However, treatment with cytochalasin D to disassemble actin filaments did not interfere with aPKC-PS induced membrane extensions. We determined by performing a quantitative membrane binding assay that aPKC-PS promoted aPKC recruitment to membranes that resulted in Src activation and subsequent tyrosine phosphorylation of several substrates including tubulin. The Src specific inhibitor PP2 blocked tubulin tyrosine phosphorylation in the binding assay as well as precluded the aPKC-PS induced MT-rich membrane extensions. These results suggest that aPKC indirectly regulates MT dynamics through Src. (This work is supported by the NIH; GM68813)

1809/B188

**Spatial Guidance of Microtubule Plus-Ends by Septin 2 in Mammalian Epithelia.**

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Regulation of microtubule dynamic instability is essential for many biological processes including mitosis, cell motility and the generation of cell polarity. The molecular mechanisms that spatially regulate microtubule dynamics are poorly understood. Septins are a conserved family of oligomeric GTP-binding proteins required for the establishment of cell polarity. Previously, we
showed that septin 2 (SEPT2) associates with a subset of microtubules, on which it facilitates the anterograde transport of Golgi-derived vesicles (Spiliotis et al., JCB 180: 295-303, 2008). Unexpectedly, we have discovered that these SEPT2-associated microtubules are also essential for the directionality of microtubule growth. In subconfluent monolayers of MDCK kidney epithelia, time-lapse microscopy of SEPT2-YFP and Eb1-dsRed revealed that microtubule plus-ends track along SEPT2 filaments with a strong anterograde bias. Retrospective staining of SEPT2-YFP/Eb1-dsRed-expressing cells for α-tubulin showed that these SEPT2 filaments colocalize with microtubules. This raised the possibility that microtubule plus ends piggyback on SEPT2-associated microtubules, which was confirmed by imaging of α-tubulin-GFP and Eb1-dsRed. Quantitative analysis of the rates of Eb1-dsRed movement showed that microtubule plus-end movement is slower on SEPT2 filaments than in the cytoplasm (12 μm/min vs. 16 μm/min). Knock-down of SEPT2 resulted in a significant increase in the mean rate of Eb1-dsRed movement and a decrease in the frequency of pausing and anterograde movement of Eb1 plus-ends when they encountered a microtubule. Remarkably, these effects resulted in two phenotypes. First, lateral movement of microtubule plus-ends was increased. Second, the number of microtubule intersections was increased resulting in a highly "entangled" microtubule network. Our results indicate that SEPT2 guides spatially the plus-ends of microtubules toward the cell periphery, which appears to be essential for the overall organization of the microtubule network.

1810/B189
Eribulin Binds at Microtubule Ends to a Single Site on Tubulin to Suppress Dynamic Instability.
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Eribulin mesylate (E7389), a synthetic analog of the marine natural compound halichondrin B, is in Phase III clinical trials for the treatment of cancer. Eribulin targets microtubules, suppressing dynamic instability through an inhibition of microtubule growth with little or no effect on shortening, resulting in mitotic arrest and apoptosis. We examined the mechanism of eribulin’s unique effect by measuring the binding affinity of eribulin for soluble tubulin and microtubules. The binding of [3H]eribulin to soluble tubulin indicated a single binding site; however, this binding is complex, possibly due to varying affinities for tubulin isotypes. Eribulin bound to preassembled microtubules with a maximum stoichiometry of ~15 binding sites per microtubule, strongly suggesting a high affinity binding site at microtubule ends. At 100 nM eribulin, the concentration that resulted in a 50% inhibition of microtubule plus-end growth [Jordan, M.A, et al., (2005) Mol. Cancer Ther 4, 1086 - 1095], one molecule of eribulin was bound per 2 microtubules, suggesting that the binding of a single eribulin molecule at a microtubule end potently inhibits its growth. This same concentration of eribulin did not suppress dynamic instability at microtubule minus ends, suggesting that eribulin primarily binds to plus ends. By electron microscopy, we examined the fine structure of microtubules incubated with 50 µM eribulin or 50 µM vinblastine. We found that eribulin does not induce significant protofilament peeling or spiraling in the manner of vinblastine; however, it does result in a slight splaying of the microtubule ends. Pre-incubation of microtubules with 2 or 4 µM vinblastine followed by addition of eribulin induced additional lower affinity eribulin binding sites, possibly at vinblastine-stabilized splayed microtubule ends. Overall the results indicate that eribulin binds to microtubule plus ends with high affinity, thus suppressing dynamic instability. Supported by grants from Eisai Research Institute and NIH (CA57291).

1811/B190
Study of the Role of Gamma-Tubulin Complexes in Microtubule Dynamics during Mitosis.
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Nucleation of microtubules (MTs) is supported by γ-tubulin, a protein component of two major complexes: the γ-Tubulin Small Complex (γ-TuSC) and the γ-Tubulin Ring Complex (γ-TuRC). Proteins of the γ-TuSC are essential for viability and assembly of a functional mitotic spindle. The
larger γ-TuRCs comprise the γ-TuSC proteins and four specific, additional proteins. γ-TuRC-specific components are considered non-essential but required for efficient mitotic progression and organization of cell type-specific MT networks (Verollet et al., 2006, J. Cell Biol. 172:517-528; Schnorrer et al., 2002, Dev Cell 3:685-696). Our recent work on interphase Drosophila cells provides evidence that the γ-TuRC is not critical for MT nucleation, but instead for the stabilization of MT plus-ends. In addition, we find γ-TuRCs localized along MTs. Here, we analyze the role of the γ-TuRC during mitosis. In Drosophila S2 cells, the RNAi of the γ-TuRC-specific protein Dgrip75 induces a delay in mitosis, increased spindle length, decreased density of spindle MTs, and longer astral MTs. Moreover, time-lapse analysis of mitotic spindles indicates that they often rotate prior to anaphase to establish the axis of cell division. on average, the angle of rotation is significantly higher in cells depleted of γ-TuRC components compared to control cells. We hypothesize that these phenotypes result from a modification in MT dynamics. Because dynamic parameters of individual MTs are difficult to quantify within the mitotic spindle, we analyzed instead the synergistic or antagonistic effects of regulators of MT dynamics on spindle assembly while depleting γ-TuRC-specific components. We started our analysis by investigating plus end-tracking proteins, +TIPs. EB1 stabilizes MTs in mitosis, as its depletion in Drosophila cells induces mainly short spindles and loss of astral MTs (Rogers et al., 2002, J Cell Biol 158:873-884). Interestingly, after simultaneous depletion of Dgrip75 and EB1, cells exhibit spindles similar to control cells. These results demonstrate for the first time that down-regulation of a specific +TIP compensates mitotic defects induced by γ-TuRC depletion.

1812/B191
Tubulin Polyglutamylation Regulates Microtubule Severing.
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Microtubules (MTs) are cytoskeletal polymers that are essential for many different cellular functions including cell division, intracellular transport and cell motility. MTs adapt to these different functions and generate specific structures by selectively interacting with different MT-associated proteins (MAPs) and molecular motors, even in a single cell. Spastin and Katanin are MT severing proteins that use ATP to induce MT breakage. They act as regulators of MT dynamic and MT polymer mass and are critical for assembly/disassembly of diverse MT arrays. Thus, MT-severing is an important process that contributes to the regulation of MT functions in mitosis, cytokinesis, ciliogenesis or axonal branching. The mechanisms that regulate the localisation and activation of these MT severing proteins have remained mostly unknown. Objectives: Here, we investigated whether polyglutamylation, a posttranslational modification of MTs, is involved in recruiting and/or activating MT severing proteins. Methods/Results: Using polyglutamylation specific antibodies in immunofluorescence, we observed that long polyglutamate side chains are found specifically on MTs in the centrosomes and on the midbody of HeLa cells, sites were MT severing is required. By changing the MT polyglutamylation level in the cells, we demonstrated that the localization and activation of the MT severing proteins is dependent on the presence of long polyglutamate side chains on MTs. The increase in severing activity of spastin in the presence of MTs that have long polyglutamate side chains was confirmed in an In Vitro MT severing assay using MTs polymerised from differently glutamylated HeLa tubulins. Conclusions: Here, we provide in cellulo and In Vitro evidences that polyglutamylation can regulate MT severing. This suggests that the specific polyglutamylation patterns found on MTs in cycling cells could play a key role in recruiting and activating MT severing proteins

1813/B192
Dissecting The Minimal Chromatin Components Required for Bipolar Spindle Assembly.
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Mitotic chromosomes generate biochemical signals that promote microtubule polymerization and spindle assembly. Chromatin itself, formed on plasmid-DNA coated beads, is sufficient to induce
spindle assembly in Xenopus egg extracts, but the molecular mechanisms underlying this phenomenon are poorly understood. We investigated the role of three different chromatin components individually or in combination using a reconstitution approach. Individual, porous microbeads 10 microns in diameter were coated with either the RCC1 nucleotide exchange factor for Ran, the mitotic kinase Aurora B, or a Kinesin 1 motor domain to mimic chromokinesins. Single RCC1 beads generated gradients of RanGTP in egg extracts that induced formation of bipolar spindles with dimensions similar to chromatin-coated beads, but at a significantly lower efficiency (20% vs. 75%). RCC1 bead spindle assembly improved dramatically upon addition of excess wild-type Ran, indicating that localized hyperactivation of the RanGTP pathway is sufficient for spindle assembly. Addition of kinesin or Aurora B to the RCC1 beads increased the efficiency of bipolar structure formation by 15% and 30% respectively. In contrast, beads coated with Aurora B alone did not generate bipolar spindles, but rather induced large microtubule arrays that lacked poles, or monopoles with astral microtubules much longer than in RCC1-induced structures. Unlike chromatin beads, RCC1 and RCC1/Aurora B beads did not remain in the center of bipolar structures, but could be stabilized by the further addition of kinesin. RanGTP-sensitive FRET probes and biochemical assays are being used to directly compare RCC1 and Aurora B activities on beads to their normal levels on chromatin. We are optimizing the ratio and levels of these factors as a key step toward complete spindle reconstitution from purified components.

1814/B193

**Microtubule Assembly by the Apc Protein Is Regulated by Importin Beta/RanGTP.**

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Adenomatous Polyposis Coli (Apc) is a tumour suppressor mutated in colorectal cancers. It is a large multidomain protein with multiple binding partners. It controls the turnover of beta-catenin and regulates microtubule dynamics in interphase and mitosis. Lack of functional Apc leads to spindle defects that contribute to chromosomal instability, a common and early feature of colorectal cancer. However, little is known about the regulation of Apc functions. Here we describe a new binding partner for Apc, importin-beta. Importin-beta is a transport factor that is involved in the nuclear import of various cargo molecules in interphase, but also contributes to spindle formation by “delivering” spindle-promoting activities to chromatin-proximal regions in mitosis. Its ability to bind cargo is regulated by the small GTPase Ran. We discovered that Apc directly interacts with importin-beta and that this interaction is inhibited by RanGTP. We mapped the importin-beta interaction sites of Apc and the regions of importin-beta that are involved in binding to Apc. We further investigated the functional consequences of this interaction and found that importin-beta reduced the ability of Apc to assemble and bundle microtubules. It also could compete with beta-catenin for Apc in vitro, but did not affect the affinity of Apc for microtubules or EB1. Furthermore, we obtained evidence that importin-beta negatively regulates the function of Apc in spindle formation. The ability of Apc to promote the formation of cold-stable spindles in Xenopus egg extract was inhibited when Apc was bound to importin-beta. In conclusion, we identified importin-beta as a direct binding partner of Apc and an important modulator of its microtubule-assembling and spindle promoting activity.

1815/B194

**The Nup107-160 Complex and γ-TuRC Regulate Microtubule Polymerization at Kinetochores.**

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In metazoan cells, the nuclear pore complex (NPC) breaks down during mitosis and many of its constituents are targeted onto spindles, including the Nup107-160 sub-complex. Nup107-160 is a stable complex that consists of nine proteins: Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Sec13, and Seh1. Nup107-160 is broadly distributed on spindles during prometaphase. It remains kinetochore-bound throughout mitosis, and shows enhanced accumulation on unattached kinetochores. We have found that Nup107-160 interacts with an active form of the γ-tubulin ring complex (γ-TuRC), an essential and conserved microtubule nucleator. We further observed that γ-TuRC localized to unattached mitotic kinetochores in a manner that requires Nup107-160. We tested whether Nup107-160 and γ-TuRC coordinately promote nucleation of microtubules near mitotic chromosomes and at kinetochores. Three observations support this idea: First, Xenopus egg extracts lacking the Nup107-160 complex or γ-TuRC failed to assemble spindles around sperm chromatin or DNA beads. Second, HeLa cells lacking Nup107-160 or γ-TuRC were profoundly deficient in kinetochore-associated microtubule nucleation. Finally, magnetic beads with immunoprecipitated Nup107-160 and γ-TuRC can induce microtubule formation in assays using purified tubulin. Notably, precipitates from Xenopus egg extracts depleted of Ran-GTP lacked this ability, suggesting that it positively regulates the function of Nup107-160 and γ-TuRC. Our findings indicate that Nup107-160 promotes spindle assembly through Ran-GTP regulated nucleation of microtubules by γ-TuRC at kinetochores. These observations suggest an important and novel relationship between the NPC and the microtubule cytoskeleton.

1816/B195
High Microtubule Growth Variability Explained by Near-Khz Microtubule Assembly Kinetics.
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Microtubules are intracellular linear polymers that dynamically grow and shorten at their ends via the stochastic addition and loss of αβ-tubulin heterodimers. The kinetics of tubulin subunit addition and loss are critical to microtubule assembly regulation by microtubule-associated proteins and therapeutic drugs. Previously, rates of tubulin subunit exchange at the ends of growing microtubules have been estimated using a linear growth theory (Oosawa, 1970), which assumes that tubulin dissociation occurs at a constant rate regardless of the free tubulin concentration (Mitchison and Kirschner, 1984; Walker et al., 1988; Chretien et al., 1995). We now find via molecular-level simulations that the tubulin dissociation rate during microtubule growth is not expected to be constant, but rather should increase with increasing tubulin concentration. This indirect effect is due to a concentration-dependent bias in simulated microtubule tip structure, as has been experimentally observed (Chretien et al., 1995). As a consequence, we predict theoretically that the published tubulin subunit addition and loss rates at growing microtubule ends In Vitro have been consistently underestimated in the literature by at least 10-fold. In addition, we find experimentally that the variance in assembly rate In Vitro is too high to be consistent with the previous low kinetic rate estimates, and conclude that tubulin addition and tubulin loss events occur at near-kHz rates (i.e. on the millisecond time scale), far faster than previously believed (0.1-0.001 kHz, i.e. 10-1000 millisecond time scale). More generally, our theoretical analysis demonstrates that the linear growth theory of Oosawa (1970) is problematic for self-assembled polymers having both lateral and longitudinal bonding interactions between subunits.

1817/B196
Cell Length-Dependent Microtubule Accumulation during Polarization.
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Breaking cell symmetry, also known as polarization, is critical to stem cell division, tumor metastasis, and neuronal development. A dramatic example of symmetry breaking is neuron
polarization, where one of the symmetrically-distributed minor processes (MPs) evolves into a single long axon. Establishing cell polarity requires that dynamic microtubules (MTs) reorganize to accumulate in the preferred direction (e.g. toward the nascent axon). MT plus ends grow outward from the centrosome towards the cell periphery by self-assembly and then stochastically switch to rapid shortening, a phenomenon known as dynamic instability (DI). A prevailing polarization model is the selective stabilization model (SSM) which hypothesizes a spatial-temporal shift towards net assembly at, or the cortical capture of, the MT plus ends in the preferred direction. Before testing the SSM experimentally, we constructed a simpler model where the DI assembly parameters are spatially and temporally constant and MTs are not cortically captured during neuron polarization. Surprisingly, this model predicts that as a neurite (MP or axon) lengthens it will naturally accumulate MTs, which we term the "length-dependent" model (LDM). In the LDM, MTs accumulate in longer neurites because the mean first-passage time for plus ends to exit the neurite via DI (i.e. via a random walk) increases monotonically with neurite length. We then experimentally tested the models by tracking MT plus end dynamics in polarizing embryonic chick forebrain neurons (ECFNs). We find that assembly dynamics remain constant in time and space during axon formation, and that MTs turn over completely in <5 minutes, consistent with the LDM and inconsistent with the SSM. In summary, polarization can occur through cell length-dependent accumulation of MTs without MT stabilization or capture. In this way, F-actin mediated cell shape and size changes can be "read-out" by dynamic MTs undergoing simple DI to ultimately break cell symmetry.

1818/B197
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Actin has been postulated to play a role in the formation and maintenance of certain types of microtubule (MT) arrays based on studies with fluorescent probes and actin filament disrupting drugs, but how actin contributes to MT organization is unknown. The preprophase band (PPB), which helps establish the future division site in plant cells, is a cortical MT array that is affected by actin-disrupting drugs. The PPB originates as a broad band of MTs during the G2 phase and matures into a narrow band during late prophase. Actin co-localizes with early but not late stage PPBs. To better understand how actin interacts with MTs during PPB development, we have investigated the ultrastructure of the cell cortex of high pressure frozen onion cotyledons by means of dual-axis electron tomography. Actin filaments are observed as 6-7 nm wide microfilaments in electron microscopical images. In interphase cells, most of the microfilaments appear randomly organized with few showing any association with MTs, which are also randomly distributed in the cell cortex. In contrast, at the onset of PPB formation, many microfilaments are seen to run parallel to MTs and to be connected to MTs via cross-bridges. Mean length of the microfilaments along MTs is about 200 nm and these microfilaments covered 40% of total length of MTs in tomographic images. The number of such MT-associated microfilaments then decreases as the PPB narrows and matures. Treatment of cells with cytochalasin D causes widening of the late PPB of MTs and re-association of microfilaments with the MTs. These observations suggest that association of actin filaments with MTs constitutes a necessary step in the PPB MT maturation process, and that MTs may also be involved in regulating the distribution of cortical actin filaments during PPB development via the formation of transient, cross-bridging-type MT-filamentous actin interactions.
Cilia and Flagella III (1819 – 1837)

1819/B198
Identification of Proteins That Regulate Intraflagellar Transport.
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Recent studies suggest that the localization of signaling molecules in cilia and the structure of cilia are regulated by the intraflagellar transport (IFT) machinery. In C. elegans this regulation may be achieved by the coordination of two kinesin-2 motor complexes used for anterograde IFT, kinesin II and OSM-3/KIF17. In the cilia middle segments IFT is mediated by OSM-3 and kinesin II together, but only OSM-3 mediates IFT in the distal segments. We recently identified DYF-5, a MAP kinase that is involved in the regulation of IFT in C. elegans. dyf-5 knockout mutants have elongated cilia, while animals overexpressing dyf-5 have shorter cilia. In animals that lack dyf-5, IFT particles are only transported by kinesin II, although OSM-3 is still present and moves along the microtubular axoneme, but at a reduced speed. In addition, kinesin II also enters the cilia distal segment (Burghoorn et al., 2007). DYF-5 belongs to a small subfamily of MAP kinases. Mammals have three DYF-5 homologues, MAK (Male germ cell-associated kinase), MRK (MAK-related kinase) and MOK (MAPK/MAK/MRK overlapping kinase). GFP tagging shows localization of all three proteins at the basal body and suggests that MAK and MRK enter the cilia. We have set up live imaging of IFT in mammalian cells. We are currently testing if RNAi mediated knockdown of MAK, MRK or MOK affects cilia length and/or IFT velocities. In order to understand the role of DYF-5 and its mammalian homologues in the regulation of IFT, we use a biochemical and a genetic approach. The biochemical approach involves In Vivo biotinylation of DYF-5 and its mammalian homologues, pull-down of biotinylated complexes and mass spectrometry to find proteins that function in the same complex as DYF-5, MAK, MRK or MOK. We are currently establishing transgenic strains that carry Bio tagged constructs in combination with a birA biotin ligase expression construct. In addition, we have performed a genetic suppressor screen in C. elegans that overexpress dyf-5 to identify mutants that suppress the cilia defects of these animals. A first screen has identified 3 suppressor mutants. We are currently mapping the mutations in these strains.

1820/B199
The Flagella Membrane of Chlamydomonas Is a Specialized Lipid Domain of the Plasma Membrane.
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Chlamydomonas is a eukaryotic biflagellate microalga. Since the flagella can be easily isolated from the cell body, this alga is ideal for biochemical studies of flagella. Cilia and flagella are essentially identical organelles; as a result, Chlamydomonas has been extensively investigated as a model for the role of cilia in human health and disease. However, almost all studies of flagella have focused exclusively on proteins. Because lipids also are likely to be important in the function of cilia and flagella, our objective is to establish the lipidome of the Chlamydomonas flagellum. Wild-type and cell wall mutant cells were grown in liquid medium under controlled culture conditions and deflagellated by treatment with dibucaine. The lipid profile of whole cells, purified cell bodies, flagella, and plasma membrane (PM) were compared by mass spectrometry. The analyses revealed that flagella, cell bodies, and PM have different lipid profiles. The most abundant fatty acids (FAs) in flagella, representing 95% of the total, were palmitic (37%), stearic (22%), γ-linolenic (21%), and oleic acids (15%). The tri-unsaturated FA γ-linolenic is enriched in flagella as compared to the PM (2%) and to cell bodies (5%). The ratio of saturated to unsaturated FAs, a measurement of membrane fluidity, in flagella was higher (1.5) than in cell bodies (0.4), but lower than in PM (7.4). Quantitative analysis of FAs showed that lauric, stearic, arachidic, and lignoceric saturated acids were enriched in the flagella two-to-four fold as
compared to cell bodies. Importantly, β-sitosterol, lyso-phosphatidic acid, lyso-phosphatidylethanolamine, phosphatidylglycerol, and six unidentified lipids were found exclusively in flagella, suggesting that the lipid composition of the flagellar membrane is distinct from that of the PM with which it is ultrastructurally continuous. These results show for the first time that the flagella membrane of *Chlamydomonas* has a distinct lipid profile from that of cell bodies and PM; that the flagella are enriched, by an unknown mechanism, with γ-linolenic acid; and that this long chain (18 carbons) triunsaturated FA might play a major role in the structure, organization, and function of flagella.

**1821/B200**

*Links between Sterols, Cilium-Generated Signaling, and Intraflagellar Transport in Chlamydomonas.*

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During fertilization in *Chlamydomonas*, plus and minus gametes adhere to each other by their flagellar adhesion molecules, the plus and minus agglutinins, thereby activating an intraflagellar signaling pathway leading to gamete activation and cell fusion to form zygotes. Previously, we showed that the intraflagellar transport (IFT) machinery is essential at an early step in the pathway, upstream of adhesion-induced tyrosine phosphorylation of a cGMP-dependent protein kinase (PKG), and that IFT complexes form higher order assemblies during signaling. In the sonic hedgehog pathway in mouse, sterols are implicated in movement of the Shh effector smoothened into the primary cilium. Here, we report studies that link sterols to flagellar adhesion-induced signaling and IFT in *Chlamydomonas*. Treatment of flagella with the sterol-binding agent methyl beta cyclodextrin (MbCD) reduced flagellar sterols by 70%, and MbCD treatment of gametes blocked flagellar adhesion-induced gamete activation and zygote formation. MbCB also blocked tyrosine phosphorylation of PKG, inhibited formation of adhesion-induced larger IFT assemblies, and inhibited a newly discovered, adhesion-activated modification of an IFT complex protein, IFT139. IFT139 is present as a single SDS-PAGE isoform in cell bodies and as two isoforms in flagella. In Vitro assays indicate that the two forms differ in their phosphorylation state. The transient conversion of the upper form to the lower form triggered by adhesion in control cells is inhibited in the sterol-depleted samples. Analysis of nystatin-resistant, sterol-deficient mutant gametes show that they are motile and that their flagella undergo normal IFT. on the other hand, they fail to express functional flagellar agglutinins and they fail to form zygotes when mixed with minus gametes. The gametes, however, are otherwise fully functional and fuse to form zygotes when artificially activated by db-cAMP. Our results indicate that sterols are essential for cilium-generated signaling in *Chlamydomonas*. Sterols are required for expression of functional flagellar agglutinins, and they are required in the pathway that couples flagellar adhesion to IFT-dependent signaling within flagella. Supported by NIH GM25661.

**1822/B201**

*Membrane Vesicle Trafficking from the Cytoplasm, To the Flagellum, and Into the Medium.*

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Cilia and flagella are sensory and motile organelles composed of a microtubule core encased in a specialized domain of the plasma membrane. Although the membranes of cilia and flagella are continuous with the plasma membrane, cells are able to localize proteins and lipids specifically to these domains by mechanisms that are not fully known. The flagellated algal model system, *Chlamydomonas reinhardti*, offers unique experimental advantages for the study of these mechanisms because all relevant membrane compartments can be readily isolated. Our objective is to characterize the trafficking pathway of membrane vesicles from the cytoplasm, to the flagellar membrane, and into the medium by isolating each vesicle population and analyzing its composition. We have obtained from cytoplasmic extracts a population of vesicles that contain proteins destined for the flagellum, such as polycystin-2 (PKD2) and components of intraflagellar transport (IFT) particles. We have also obtained highly purified membrane preparations from both
the flagella and cell bodies of Chlamydomonas and found that several membrane proteins including PKD2 and a voltage-dependent calcium channel (CAV2) are greatly enriched in the flagellar membrane. These data suggest that there is a diffusion barrier or gate between the flagellar and cell body membranes. Using fluorescent ConA, which labels flagellar membrane glycoproteins, we have been able to follow, in vivo, the active movement of membrane proteins to the tip of the flagellum by IFT and their secretion on membrane vesicles into the medium. We have also documented by transmission electron microscopy the pinching-off of these vesicles at the flagellar tip. The vesicles that are secreted from the flagella into the medium contain an enrichment of a subset of flagellar polypeptides such as tubulin, 14-3-3, PKD2 and high molecular weight ubiquitinated proteins. This secretion probably occurs in almost all cilia and balances the addition of membrane at the base of the cilium. At present, we are investigating the possible signaling functions of these secreted vesicles during the flagella-dependent mating process of Chlamydomonas. Supported by NIH grant GM14642.

1823/B202

**Chlamydomonas CEP290 Is a Transition Zone Protein Required for Normal Intraflagellar Transport and Flagellar Assembly.**

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Mutations in CEP290 cause cilia-related disorders that range in severity from retinal disease, with other organ systems mostly unaffected (Leber's congenital amaurosis), to pleiotropic defects in multiple organ systems including the kidney and the brain, the most severe of which is perinatal lethal (Meckel syndrome). CEP290 has been reported to localize to centrosomes, basal bodies, the nucleus, and the photoreceptor connecting cilium, yet the molecular function of CEP290 remains poorly understood. We sought to address this deficiency using *Chlamydomonas*, which has numerous characteristics that make it an ideal model system for elucidating the basic function of highly conserved ciliary and basal body components. The *Chlamydomonas* CEP290 homologue, encoded by *POC3*, is highly conserved (BLAST E = 5e-27). A PCR screen of a library of *Chlamydomonas* insertional mutants identified a strain in which all but the first 3-5 exons of the gene encoding *Chlamydomonas* CEP290 are deleted. The CEP290 mutant cells display defects in hatching after mitosis, flagellar assembly, and motility. The mutant phenotype cosegregated with the deletion, and transformation of the mutant with genomic DNA encoding wild-type or epitope-tagged CEP290 rescued the mutant phenotype, confirming that the defects are due to the deletion in CEP290. An antibody to *Chlamydomonas* CEP290 confirmed that the mutant lacks CEP290 and demonstrated that CEP290 localizes to the transition zone of flagella. Ultrastructural analysis of CEP290 mutant cells revealed defects in the connections between the outer doublets of the transition zone and the flagellar membrane. Some flagella also have abnormal bulges filled with electron dense, amorphous material. Comparison of wild-type and CEP290 mutant flagella by western blot revealed that the mutant flagella contain increased levels of IFT complex B proteins and BBS4, and decreased levels of the IFT complex a subunit IFT139 and the polycystin-2 homologue PKD2. The results suggest that CEP290 is a component of the membrane-microtubule connections of the transition zone and functions in the assembly of IFT complex a with complex B.

1824/B203

**Chlamydomonas IFT25 Is Involved in Regulating the Expression Levels of the IFT Complex and Axonemal Proteins.**

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Intraflagellar transport (IFT), the bidirectional movement of IFT particles within flagella, is required for flagellar assembly in eukaryotic organisms. IFT particles are composed of at least 18 proteins that are organized into complexes a and B. Our previous study (Wang Z, Fan Z-C, Williamson SM, Qin H 2009 PLoS ONE 4(5): e5384) showed that Chlamydomonas IFT25 is a 25-KD phosphorprotein component of the IFT complex B and interacts directly with IFT27, a small Rab-
like G-protein component of the IFT complex B. However, the role of IFT25 in flagellar assembly remains uninvestigated. In this study, partial knockdown of the IFT25 expression by RNA interference greatly reduces the levels of other IFT complex α (IFT139) and B (IFT172, IFT81, IFT72, IFT46, IFT27 and IFT22) proteins indicating that IFT25, different from IFT proteins IFT46 and IFT88, is critically involved in stabilizing both IFT complexes. More interestingly, the expression levels of the outer dynein arm intermediate chain 69 (IC69) was also greatly reduced in the IFT25-knockdown strain demonstrating the IFT25 also plays a role in regulating the expression of the axonemal proteins. Consequently, the IFT25-knockdown cells have short flagella. Taken together, IFT25, similar to IFT27, is essential in flagellar assembly and specifically required for maintaining the stability of both IFT complexes. IFT25 also plays a role in modulating the expression of the axonemal protein IC69. Currently, the role of IFT25 phosphorylation in flagellar assembly is under investigation.

1825/B204
The Intraflagellar Transport Particle Injector Controls Eukaryotic Flagellar Length.
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The intraflagellar transport (IFT) particle injector controls eukaryotic flagellar length. The injector works by restricting the availability of new material for growth of the organelle, analogous to a fuel injector controlling the speed of a single piston engine by limiting fuel. Using quantitative TIRF microscopy and computational image processing we measure GFP-tagged IFT proteins KAP and IFT27 in Chlamydomonas reinhardtii flagella over a range of cellular and flagellar states (i.e. regenerating cell vs steady state cell and short flagellum vs long flagellum). From measuring the IFT particles in the flagellum, we then back-calculate the behavior of the IFT particle injector. We then derive mathematical models for the system that controls the IFT particle injector, finding that our data are consistent with a two-state time of flight model and not a diffusional signal or a constant IFT particle number model as previous studies have suggested. These results indicate that the group of proteins responsible for the injector behavior includes a two-state protein, such as a GTPase, that travels the length of the flagellum to measure its length. A mutant in this putative protein with either a constitutive excited state or a constitutive ground state would then have abnormally long or abnormally short flagella respectively. Our results further indicate that the flagellar length is set by the flagellum rather than the cell, which implies that the organelle can self-regulate, to some extent independent of the cell.

1826/B205
The PP2A B-Subunit Protein Is Absent in the Chlamydomonas Paralyzed Flagellar Mutant pf4 and Is Required for Targeting of the PP2A Holoenzyme to the Axoneme.
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Previously, we localized the A- and C-subunits of the protein phosphatase PP2A to the outer-doublet microtubules of the Chlamydomonas axoneme (Yang et al., 2000). We postulated that the axoneme must also contain a PP2A regulatory B-type subunit, responsible for localizing the heterotrimeric PP2A holoenzyme. To test this, we identified an axonemal PP2A B-subunit in the Chlamydomonas flagellar proteome (Pazour et al., 2005). The PP2A B-subunit gene model, (185509, JGI, Version 3), predicts a protein that is homologous to other members of the WD-repeat containing, PR55 family of B-subunits. Immunoblots confirmed that the B-subunit is an axonemal protein that is extractable in 0.6M NaCl. The B-subunit gene maps near the pf4 locus on linkage group I. We confirmed that pf4 is defective in the gene for the PP2A B-subunit; pf4 has a base pair deletion / substitution that is predicted to result in a premature stop codon. The pf4 mutant cells have full-length flagella, display a smooth, medium velocity swimming phenotype and are defective in phototaxis. This phenotype is similar to mutant cells lacking inner arm I1 / f-dynein. Immunoblots revealed that B-subunit protein is absent in pf4 mutant cells and axonemes. Additionally, the PP2A C-subunit is absent in the pf4 mutant axonemes. These results
demonstrate that the B-subunit of PP2A is required for targeting the holoenzyme to flagella and axonemes and that PP2A is required for regulation of flagellar motility.

1827/B206
Rer1p, a Quality Control Receptor in the Early Secretory Pathway, Is Required for the Maintenance of Functional Cilia.
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Proteome analysis of isolated cilia revealed hundreds of proteins essentially dedicated to cilia organization and function. However, hitherto, very few proteins are known involved in the targeting of membrane proteins to the cilium. More precisely, how membrane proteins, after their biosynthesis in the endoplasmic reticulum (ER), reach the ciliary membrane remains largely unexplored. Unexpectedly, we recently identified the early cargo receptor Rer1p as being critically involved in ciliogenesis and cell polarity. Rer1p operates in the quality control of membrane proteins during transport between the ER and Golgi complex. Rer1p might therefore represent a candidate protein linking early biosynthetic sorting with trafficking to the ciliary membrane. Using zebrafish as a model system we demonstrate that zfRer1p is highly expressed in ciliated organs, such as olfactory pit, otic vesicle, neuromasts of the lateral line and pronephros. Moreover, morpholino-mediated knockdown of its gene in zebrafish embryos results in a typical ‘curly’ phenotype found in mutants with defective cilia. For instance, zebrafish morphants present with bent body axis, laterality defects and impaired development of the lateral line organ. Strikingly, downregulation of Rer1p was sufficient to cause embryonic deafness, pointing to essential role of this protein in proper development and function of the inner ear. Most of these features are commonly observed in patients suffering from cilia-related disorders, known as ciliopathies. In addition, we observed that Rer1p knockdown in LLC-PK1-CL4 (porcine kidney epithelial) cells severely reduces cilia length, while it increases the amount of acetylated α-tubulin, a stable form of tubulin found in cilia. Similar increases in acetylation is observed throughout the development of ciliated cells in zebrafish. This already offers a first hint towards revealing the possible mechanism of Rer1p in ciliogenesis. In conclusion, we describe here the first involvement of a quality control mechanism of early biosynthetic compartments in the morphogenesis or maintenance of functional cilia.

1828/B207
Joubert Syndrome-Associated ARL13B Associates with the Ciliary Membrane and Is Required for the Proper Localization and/or Transport of Ciliary Proteins in C. elegans.
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Joubert syndrome (JS) and JS-related disorders (JSRD) are clinically heterogeneous, characterized by cerebellar malformation and hypervariable phenotypes such as cystic kidneys, retinitis pigmentosa and polydactyly, and emerging evidence indicates that JS/JSRD disorders are caused by defects in primary cilia. Using mammalian tissue culture and C. elegans models, we investigated the molecular basis of ARL13B/ARL-13 function, a cilia-specific G-protein which causes classical JS and is required for vertebrate cilium formation and sonic hedgehog (Shh) signaling. First, by employing immunostaining and GFP-tagging, we found that ARL13B and ARL-13 localize predominantly to the proximal regions of cilia on C. elegans sensory neurons and MDCKII cells. Disruption of an N-terminal palmitoylation modification motif causes mislocalization of ARL13B and ARL-13 to the cytosol, indicating that the wild-type G-protein associates with the ciliary membrane. Next, using a deletion allele, we conducted functional analyses of C. elegans arl-13 and found that mutants possess defective cillum morphologies and ultrastructures, as well as defects in the ciliary localizations of transmembrane proteins. Although the ciliary localizations of intraflagellar transport (IFT) proteins are unaffected in arl-13 mutants, overexpression of IFT transgenes enhances the cillum integrity defects of ARL-13-deficient cilia and destabilizes anterograde IFT. Finally, we find that arl-13 interacts synthetically with other ciliopathy/ciliary
transport-associated genes (e.g., nph-4, dyf-5) in maintaining cilium integrity, suggesting that arl-13 functions in a distinct parallel/converging genetic pathway. Together, these data implicate a unique role for ARL13B/ARL-13 at the proximal ciliary membrane, where it is required for the localization and/or transport stability of ciliary transmembrane proteins and IFT assemblies. We propose that disruption of these functions underlie the molecular aetiology of ARL13B-associated JS and Shh signaling defects in mice.

1829/B208
A Flagellar AKAP Anchors Four Non-PKA RIIa Clan Members for Integrated Functions.
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The RIIa domain in the regulatory subunit RII of cAMP-dependent protein kinase (PKA) docks the holoenzyme to an amphipathic helix in A-Kinase Anchoring Proteins (AKAPs). Numerous AKAPs have been identified for their ability to bind RII in vitro, presumably localizing the kinase near intended substrates for tight regulation spatially and temporally. Interestingly, RIIa and a homologous domain, DPY-30 are categorized in the RIIa-clan superfamily which includes nearly 400 molecules from diverse organisms. Aside from these docking domains, most members contain diverged sequences irrelevant to PKA. Some are related to nucleotide metabolism or calcium signaling, while others are short and inconspicuous. For instance, DPY-30, the namesake of the domain is a small yet critical subunit in various chromosome modification complexes, suggesting that the scope of this superfamily is much broader. To investigate the general principle of RIIa clan superfamly, we take advantage of Chlamydomonas radial spoke (RS) complex, a putative mechanochemical transducer that controls flagellar beating. Among RS constituents are two non-PKA subunits with a RIIa domain, two calmodulin-binding subunits with a DPY-30 domain and a spoke AKAP (RSP3) that is dimeric inherently. The two RIIa RSPs along with a subsets of RSPs are absent in truncated RSP3 mutants lacking the RIIa-binding amphipathic helix. Contrarily, the two DPY-30 RSPs and the bulbous spokehead modules are absent in mutants lacking the distal region of RSP3. All mutants are paralyzed. These results suggest that RSP3 forms the RS scaffold with two distinct sites anchoring the two RIIa clan domains and their associated effector mechanisms for integrated mechanochemical transduction. We propose that A-kinase anchoring protein (AKAP) represents a class of the RIIa-clan anchoring proteins (RAPs) that actually targets a wide range of critical functions.

1830/B209
Cooperation of AP1 Clathrin Adaptin and RAB-8 in Protein Transport to Cilia and Cilia Morphology Determination.
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Clathrin and clathrin adaptor (AP) complexes mediate protein sorting and vesicular trafficking between intracellular membrane compartments and the plasma membrane. One such compartment is the ciliary membrane, which extends from the surface of most vertebrate cells. Although previous reports in C. elegans have ascribed a function for AP-1 in ciliary protein transport, the molecular basis of AP-1-mediated ciliary formation and polarised protein targeting to cilia has not been addressed in detail and components that associate with AP-1 in delivery of ciliary proteins remain unknown. Using the C. elegans model, we show that the clathrin heavy chain (CHC-1) and AP-1 complex components (UNC-101, APS-1) are required for determining cilia morphology, positioning and ultrastructure, and for mediating vesicle transport of transmembrane proteins (ODR-10) to cilia. AP-1 functions independent of intraflagellar transport (IFT), since IFT proteins display normal ciliary localizations and motilities in unc-101 mutants, and the ciliary localisation and dendritic motilities of ODR-10 are unaffected in IFT mutants. In contrast, unc-101 and RAB-8(Q67L) (GTP-locked RAB8) mutations both prevent the formation of ODR-10 transport vesicles, cause ODR-10 to become trapped at the plasma membrane, and suppress AWB cilia membranous fan formation in grk-2 mutants. Consistent with related roles for RAB-8 and AP-1 in membrane trafficking to cilia, GFP-tagged RAB-8, UNC-101 and ODR-10 possess overlapping
subcellular localizations. Together, our results suggest that AP-1 and RAB-8 cooperate in a CHC-dependent manner to coordinate membrane trafficking to cilia.

1831/B210

**Ttc26, the Vertebrate DYF-13 Homologue, Undergoes IFT and Is Required for Cilia Formation and Maintenance.**

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The primary cilium is a ubiquitous, non-motile organelle that protrudes from the cell surface like an antenna. Recent studies have shown that the primary cilium acts as a sensory organelle to detect a wide variety of extracellular signals. Ciliary defects have been implicated in a diverse spectrum of diseases including retinal degeneration, polycystic kidney disease, left-right asymmetry defects and hydrocephalus. Primary cilia are assembled and maintained by the process of intraflagellar transport (IFT), a highly conserved mechanism in almost all eukaryotes. We analyzed the ciliary protein Ttc26, which is the vertebrate homologue of DYF-13, one of dye-filling defective mutants in _C. elegans_. GFP-labeled Ttc26 localized to the basal bodies and primary cilia of IMCD3 mouse kidney cells. In addition, we observed the IFT-like movement of Ttc26-GFP within primary cilia. Knockdown of Ttc26 in zebrafish embryos produced short cilia within Kupffer's vesicle and produced typical cilia-related defects including pronephric cysts, hydrocephalus, and the randomization of left-right asymmetry. We hypothesize that Ttc26 is an IFT protein that is required for cilia formation and maintenance.

1832/B211

**Trafficking of KIF17 into Primary Cilia Is Regulated through a Ran-GTP Gradient.**

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The biogenesis and maintenance of cilia and flagella is driven by intraflagellar transport (IFT) and the concerted action of two Kinesin-2 family members, the heterotrimeric KIF3A/3B/KAP complex and the homodimeric KIF17 motor. How Kinesin-2 motors bind to their cargoes and gain access to the cilium are poorly understood. When expressed in mammalian cells that generate primary cilia, KIF17 motors localize to the distal tips of cilia, and this accumulation requires the presence of the KIF17 tail domain. Truncated forms of KIF17 that include only the tail domain localize to nuclei as well as cilia, suggesting similar biochemical cues exist for both nuclear and ciliary localization. We identify the sequence KRKK in the KIF17 tail as necessary and sufficient for ciliary targeting, demonstrating that this sequence functions as a ciliary localization signal (CLS). To explore parallels between nuclear and ciliary import, we investigated whether ciliary trafficking of KIF17 is regulated by a Ran-GTP gradient across the ciliary transition zone. Overexpression of constitutively active Ran-G19V, which disrupts the ciliary Ran-GTP gradient, abolished KIF17 localization to cilia. Furthermore, we show that KIF17 interacts with importinβ2 and that this interaction is abolished by mutation of the CLS or the presence of purified Ran-G19V. Taken together, we propose a model for ciliary targeting of KIF17 in which the CLS interacts with an importin complex that shuttles the motor through ciliary transition fibers. The high levels of Ran-GTP in the cilium cause dissociation of the KIF17-importin complex, freeing the motor for IFT. Our results indicate that Ran plays a global role in regulating cellular compartmentalization by controlling the shuttling of protein complexes between cytoplasmic, nuclear, and ciliary compartments.

1833/B212

**The Cytoplasmic Tail of Fibrocystin Contains a Ciliary Targeting Sequence which Interacts with the Small G protein Arf4.**

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Primary cilia are found on nearly every vertebrate cell type and play vital roles in the development of mammals and in the etiology of diseases such as polycystic kidney disease and blindness. It is thought that the major function of these organelles is to perceive cues from the extracellular environment and communicate them to the cell body. The sensory functions of primary cilia rely on receptors and other membrane proteins localized to the ciliary membrane but little is known about how proteins are specifically targeted to this domain. To further our understanding of this process, we dissected the ciliary targeting signal (CTS) of fibrocystin, the product of the gene defective in human autosomal recessive polycystic kidney disease. The fibrocystin CTS is an 18 amino acid motif localized in the cytoplasmic tail. This 18 residue motif is sufficient to target GFP to cilia. We hypothesize that the CTS functions by interacting with sorting or trafficking machinery and tested for interactions with a battery of proteins implicated in trafficking to the ciliary membrane. The CTS failed to interact with most proteins but did bind the small G proteins Arf4 and Rab8, and the BBS5 subunit of the BBSome. Since Arf4 also binds the CTS of opsin (Deretic et. al. Proc Natl Acad Sci U S A. 2005), Arf4 may play a role in directing membrane proteins to the ciliary compartment. To address this idea, Arf4+-/- gene trap cells were used to create an Arf4 mutant mouse. Arf4 null mice die embryonically at mid gestation with phenotypes that include growth restriction and a failure to turn. Nodal cilia are present but are shorter than normal suggesting a role for Arf4 in ciliary assembly. However, left/right patterning is not perturbed suggesting that the nodal cilia are functional in breaking symmetry of the early embryo. Current research is focused on determining the role of Arf4 in mouse development and trafficking of the fibrocystin CTS to the cilium.

1834/B213
Regulated Flagellar Localization of the Membrane-Associated Protein Sag1 in Chlamydomonas.
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Signals generated within cilia can regulate the movement of membrane proteins into the organelle. In mammalian cells, binding of sonic hedgehog to ciliary Patched triggers movement of Smoothened onto the cilium. Our laboratory studies regulated movement of the Sag1 agglutinin protein from the cell body to the flagella in mt+ gametes of the biflagellated green alga Chlamydomonas. Bioassays have shown that in resting gametes ~80% of Sag1 is in an inactive pool on the plasma membrane of the cell body and 20% is present and active on the flagella. Binding between flagellar Sag1 on mt+ gametes and its cognate receptor Sad1 on flagella of mt-gametes causes flagellar adhesion and activation of a cilium-generated signaling pathway. As part of a positive-feedback mechanism, pathway activation triggers mobilization of additional Sag1 from the cell body to the flagella where it becomes active. Whether Sag1 molecules traffic between the two pools in resting gametes is unknown. Here, we report use of new tools to study regulation and properties of Sag1. We show that Sag1 bearing an HA epitope at its C-terminus rescues flagella adhesion in the sag1, flagellar adhesion mutant, imp9. Immunofluorescence of resting Sag1-HA rescued imp9 mt+ gametes shows that, as expected, the protein is present at the cell body and the flagella. The cell body pool is localized at the apical end of the cell near the basal bodies. Interestingly, flagellar Sag1 is unevenly distributed and enriched at the distal 1/2 of the organelle. These results indicate the presence of a mechanism for anchoring Sag1 on flagella, and suggest that in resting cells Sag1 molecules in the cell body and flagellar compartments are prevented from intermingling.

1835/B214
Primary Cilia of Corneal Endothelial Cells Express Core Planar Cell Polarity Proteins and Are Required For Hexagonal Pattern Determination.
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The corneal endothelium (CE), a monolayer of epithelial cells that forms a hexagonal mosaic on Descemet’s membrane, plays an essential role in the physiology of the cornea, maintaining its transparency by regulating corneal hydration. Although the hexagonal pattern of this epithelium is critical for its function, the mechanisms involved in its determination are largely unknown. Several studies report the presence of a primary cilium on a subpopulation of corneal endothelial cells (CEC) in vertebrates, but its function is unknown. We have studied CEC ciliary dynamics during postnatal development. By using immunofluorescence or scanning electron microscopy, a primary cilium was detected on all CEC of mice going from 2 to 15 days of age. However, in CEC of older mice the cilium progressively disassembled starting from the periphery towards the center of the cornea. By day 30-35, primary cilia were completely absent from all CEC but intraflagellar transport protein IFT74/72 remained associated to the centrosome. Resorption of the cilia correlated with a progressive formation of hexagonal CEC pattern. Early after birth CEC appeared as irregular polygons with variable number of sides and no detectable acetylated microtubules in the cytoplasm. By day 30-35 CEC reached a more regular hexagonal shape with acetylated microtubules forming a circular bundle around the nucleus. To determine whether the primary cilium was involved in hexagonal patterning of CE, we analyzed corneas from 4.5d, 6.5d and 9.5d wild-type and ift88orpk/orpk mice as well as corneas transduced In Vivo with a lentivector expressing a siRNA specific for IFT88. While all the CEC of wild-type mouse were ciliated, few areas of CE from orpk mice or lentivector transduced mice presented short or completely ablated cilium. within these areas CEC were disorganized, ZO-1 mislocalized, and acetylated tubulin accumulated in CEC cytoplasm suggesting an active role of cilia in CE patterning determination. In addition, we have found that frizzled 3, involved in PCP in vertebrate, localizes on the CEC ciliary membrane. The above findings suggest a role of the CEC cilium in mediating planar cell polarity signaling during CE morphogenesis.

1836/B215
The Novel Cilia Protein DAF-25 Is Required for Guanylyl Cyclase Localization In Chemosensory Cilia.

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In harsh conditions, the nematode Caenorhabditis elegans forms a non-aging, resistant larval diapause state known as the dauer larva. Olfactory sensation modulates the TGF-β and insulin signaling pathways to control this developmental decision. Four mutant alleles of daf-25 (abnormal Dauer Formation) were isolated from screens for new temperature-sensitive mutants that form dauer larvae constitutively, even in favorable environments. daf-25 mutants are defective in sensing osmotic gradients and multiple chemo-attractants. The daf-25 mutations are suppressed by daf-10/IFT122 but not daf-6/PTCHD3. This implies that DAF-25 function requires the sensory cilia because DAF-10 is required for cilia formation, whereas DAF-6 is required for formation of the sensory channel and exposure of the ciliated sensory neurons to the environment. These non-motile cilia form the afferent sensory ends of the sensory neurons. In daf-25 mutants, the ciliated sensory neurons stain normally with lipophilic dye and they show wild type cilia structure in electron micrographs, indicating no cilia structural defects. A functional DAF-25::GFP translational fusion protein localizes to the sensory cilia. The phenotype, epistatic order and expression profile of daf-25 are similar to daf-11, which encodes a membrane-bound guanylyl cyclase. Indeed, we show that DAF-25 is required for proper DAF-11 localization to the cilia using a functional DAF-11::GFP translational fusion. This may be a specific interaction because daf-25 mutations do not affect the normal localization of CHE-11/IFT140, CHE-2/IFT80, TAX-4/CNGA1 and OSM-9/TRPV4 to the cilia. Normal IFT (IntraFlagellar Transport) speed was observed for the
middle and distal segments, indicating no defect in core IFT. Accumulation of DAF-25::GFP was observed in che-11 mutants, which are defective in retrograde IFT, indicating DAF-25 is transported by IFT. Expression of the DAF-25 ortholog was shown in the bovine retina by Western blot and in the mouse retina by RT-PCR. The murine ortholog was shown to interact with the retinal guanylyl cyclase GC1 by co-immunoprecipitation. We show a novel, conserved cilia protein required for guanylyl cyclase localization to the cilia.

1837/B216
Stable Adhesions Form between Cilia of Mammalian Cells.
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Primary cilia extend from the cell surface of many different types of cells, including the apical plasma membrane of polarized epithelial cells. From this vantage, cilia are well poised to sample the extracellular environment. Primary cilia have been shown to passively sense osmotic changes, specific signaling molecules and changes in fluid flow rates. Active cilia sensing, through direct contact, has been well characterized during the mating of Chlamydomonas reinhardtii. In this process direct contact of flagella causes a signaling cascade that brings about the formation of mating structures. However, active cilia functions in mammalian cells have not been described. Here we characterize contact between cilia of canine kidney epithelial cells (MDCK cells) using live and fixed cell imaging. We report stable and prolonged adhesion of cilia from two or more cells. To test for signaling through cilia adhesions we compared localization of several transcription factors, such as Gli proteins and beta-catenin, in cells with or without cilia contact; we found no differences. We performed several experiments to try to disrupt the cilia adhesions in an effort to better understand the mechanism of adhesion. These cilia-cilia interactions are resistant to protease treatment, disruption of disulfide bonds and calcium chelation, which rules out several classes of cell adhesion molecules, such as Cadherins. From these studies we conclude that like cilia in other systems, mammalian cilia make direct contact with other cilia and have the potential to participate in active sensing of the extracellular environment. Future work will unveil the physiologic relevance of these observed cilia interactions.

Centrosomes II (1838 -1854)

1838/B217
Cryo-EM Structure of the Microtubule Nucleating γ-Tubulin Small Complex in Ring-like Assemblies.
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Microtubules are nucleated in vivo by complexes containing γ-tubulin. The core of the microtubule nucleating machinery, conserved in all eukaryotes, is the γ-tubulin small complex (γ-TuSC), a 300 kDa heterotetramer containing two copies of γ-tubulin. In most eukaryotes multiple γ-TuSCs assemble with a number of other proteins into large γ-tubulin ring complexes (γ-TuRCs); budding yeast, however, have only γ-TuSC and lack the other γ-TuRC-specific components, providing the ideal case to study a minimal nucleating complex. Here we show that purified γ-TuSCs have an intrinsic propensity to associate into γ-TuRC-like ring structures in the absence of any of the γ-TuRC-specific proteins. The amino terminal domain of Spc110p, which binds γ-TuSC to the nuclear face of the spindle pole body, is shown to stabilize lateral γ-TuSC interactions to such an extent that elongated filaments of assembled γ-TuSC are formed. We have determined a moderate resolution cryo-EM structure of these filaments. Within the filaments, the γ-tubulins are presented in an arrangement very similar to a single ring of tubulin in the microtubule, and the plus ends of the γ-tubulins are their only surfaces available to make microtubule-like contacts with αβ-tubulin. The helix has 6.5 γ-TuSCs, or 13 γ-tubulins, per turn, matching the predominant 13-protofilament organization of microtubules in vivo. These features of the γ-TuSC assembly offer
strong support for models of nucleation that suggest γ-tubulin complexes provide a template for microtubule growth. We propose that a single turn of the γ-TuSC filament is the equivalent of the core of γ-TuRC, suggesting that the γ-TuRC-specific proteins are not required for forming the nucleation template but likely play roles in regulating γ-TuRC activity and localization. The stabilization of γ-TuSC interaction by Spc110p suggests a mechanism for regulating nucleating activity by keeping γ-TuSC assemblies localized at the spindle pole body.

1839/B218 ABSTRACT WITHDRAWN

1840/B219 Drosophila Stem Cells Display Asymmetry in Centrosome Function.  
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Centrosomes transition between stages of low and robust activity as the cell progresses through the cell cycle. In interphase, centrioles recruit a relatively small amount of pericentriolar material (PCM) and, in turn, nucleate and anchor fewer microtubules (MTs). In mitosis, centrosomes “mature” by recruiting more PCM, nucleating more MTs and forming robust asters needed for spindle assembly. In Drosophila, we found that the centrosomes in interphase have exaggerated the maturation cycle by reducing the amount of PCM at the centriole to below detectable levels in interphase. This is seen both in tissue culture cells and in a number of cell types in the animal. Analysis of neural stem cells (neuroblasts, NB), however, reveals a specialized centrosome cycle that does not follow this canonical Drosophila maturation cycle. As NBs exit mitosis, the two centrioles within the NB centrosome disengage. One centriole remains stationary and maintains some associated PCM to form the “dominant centrosome,” which organizes MTs and resides in a fixed location. The other centriole sheds all its PCM and MTs, becomes mobile, moves to a position opposite the nucleus and only becomes active when the NB enters prophase. We propose that NBs have developed such a mechanism to tightly control the spindle axis, which is needed to properly cluster the daughter ganglion mother cells. To test the hypothesis that centrosome asymmetry is absolutely required for asymmetric division we have attempted to activate the inactive centrosome using a modified Polo kinase probe that is specifically targeted to centrioles. This probe can activate centrioles In Vitro and we are currently testing its function in vivo. To test the hypothesis that centrosome asymmetry and distal maturation have been adopted by all stem cells we are performing live cell imaging of the male germline stem cells (mGSCs). Our data suggests that mGSCs employ the same asymmetric centrosome maturation mechanism. We are also testing the possibility that an “immortal” centriole with unique properties is present in each stem cell, which could explain the observed asymmetric behavior in these cells.

1841/B220 Trichoplein Regulates Cilia Assembly and Microtubule Organization.  
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Centrosome is a major microtubule-organizing center in animal cells, regulating cell motility, adhesion and polarity. The mother centriole contributes to nucleation of microtubules, assembly of primary cilia and microtubule anchoring. However, little is known regarding the mechanisms that govern cilia assembly and microtubule organization. Here, we show that trichoplein, keratin binding protein, controls cilia assembly program and microtubule anchoring in centrosome. We show that trichoplein is a centrosomal protein localized at the distal end of the mother centriole in the epithelium of small intestine and some cell lines. In contrast, trichoplein was diminished at the ciliated mother centrioles by serum starvation. Depletion of trichoplein promotes primary cilia formation with serum, and overexpression of trichoplein suppresses cilia assembly. Moreover, we demonstrated that trichoplein associates with and controls activation of mitotic regulatory kinase Aurora-A for cilia disassembly, independently Odf2β/Cenexin1, scaffold protein in centrosome.
Additionally, depletion of trichoplein disorganized microtubule nucleation through ninein from the appendages of the mother centrioles. We propose that trichoplein is a key player at the mother centriole for the dynamic reorganization of cytoskeletal structures by altering the phosphorylation status of Aurora-A.

1842/B221
A Role for Beta-Catenin in Extra Centrosome Assembly.
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Centrosomes have a dominant role in spindle assembly in mitosis, and produce multipolar spindles when present in abnormal numbers. Despite the importance of centrosomes in cell division, the assembly of extra centrosomes, a hallmark of many cancers, is poorly understood. The oncogene β-catenin, known for its role in cell adhesion and Wnt signaling, has been shown to function in centrosome separation as a substrate for the kinase Nek2 (Bahmanyar et al., Genes &Dev. 22, 91-105, 2008). Increased levels of β-catenin and Nek2 are common in many types of cancers, and deregulation of Nek2 activity causes centrosome amplification. We found that expression of a mutant β-catenin, similar to that found frequently in cancer cells, results in extra centrosomal structures. Interphase cells expressing this mutant β-catenin have functional and non-functional centrosomal structures. Non-functional structures contain γ-tubulin and centrin but not pericentrin, and have increased amounts of PCM-1, a centriolar satellite protein involved in delivery and assembly of centrosomal components. Live cell imaging of PCM-1 and centrin revealed an immobile population of PCM-1 satellites that were associated with centrin aggregates and resembled de novo centrosomes. Furthermore, the mobile pool of γ-tubulin was increased at original centrosomes in cells expressing mutant β-catenin. Removal of the mutant β-catenin allele from cancer cells reduces the ability of these cells to form extra non-functional centrosomal structures. Taken together, these results suggest a pathway involving β-catenin in extra centrosome assembly, and provide a potential mechanism by which mutant β-catenin promotes cancer progression.

1843/B222
Determinants of Rear Polarization of MTOC in Migrating Arterial Neointimal Smooth Muscle Cells: PKC, ARPC5, and RHAMM.
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The migration of smooth muscle cells (SMCs) from the media toward the intima occurs in atheromatous plaque formation and after angioplasty or stent application. Most neointimal SMCs cultured from injured arteries or migrating from media to neo-intima are rear polarized, while most migrating medial SMCs cultured from uninjured arteries are anterior polarized. Using a phosphoproteomics screen and mass spectrometry we identified 6 proteins with higher phosphorylation levels in neointimal SMCs than medial SMCs. After PKC inhibition in neointimal SMCs, ARPC5 and RHAMM were less phosphorylated compared to controls, and most cells were anterior polarized and migrated slower. Knockdown of ARPC5 and RHAMM by siRNA showed that they are required for the rear polarity and migration of neointimal SMCs. PKC, ARPC5, and RHAMM are involved in lamellipodia organization, and ARPC5 and PKC are required for the organization of a polygonal actin net above nuclei. Our study demonstrated novel roles for PKC, ARPC5, and RHAMM in cell polarity and migration of neointimal SMCs.

1844/B223
Cancer Cell Division Mechanism: How Cancer Cells Divide in the Presence of Too Many Centrosomes.
Precise control of centrosome number is crucial for bipolar spindle assembly and accurate transmission of genetic material into daughter cells. However, many cancer cells are predisposed to catastrophic multipolar mitoses due to the presence of extra centrosomes. Interestingly many cancer cells cluster their extra centrosome, thereby enabling bipolar division. Our genome-wide RNAi screen in Drosophila S2 cells allowed the first comprehensive identification of the genetic requirements for the process of centrosome clustering. In addition, follow up studies in mammalian cancer cells and live cell imaging have demonstrated that multiple redundant mechanisms facilitate the clustering of supernumerary centrosomes. Centrosome clustering is achieved by a combination of spindle-intrinsic microtubule (MT) binding forces (such as the MT motor Kinesin-14/Ncd/HSET) and actin-regulating forces at the cell cortex whose distribution is regulated by the interphase adhesion pattern. In addition, the spindle assembly checkpoint (SAC) ensures that cells have enough time to cluster extra centrosomes before anaphase onset. Moreover, we have shown that disrupting centrosome clustering by knockdown of the nonessential kinesin HSET can selectively kill cancer cells with extra centrosomes, validating it as a clinical strategy. Current work utilizes isogenic cell lines that differ only in centrosome numbers, allowing a direct test of selective genetic requirements for bipolar division in cancer cells. In cells harboring overduplicated centrosomes in Doxycycline-inducible manner, the inhibition of HSET by siRNA or the inhibition of Mps1 (abrogating SAC) by the treatment of novel, selective small molecule inhibitor of Mps1 both resulted in catastrophic multipolar divisions in cancer cells with extra centrosomes. We are currently investigating the detailed molecular mechanisms of this complex process that is conserved in different organisms.

1845/B224
Sam68 Is Involved in Centrosome Duplication and Its Localization On Centrosome Is Regulated by Ran-Crm1.
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Objective: Our work is trying to explore the mechanism of Sam68, NPM and geminin in the centrosome duplication regulation. Methods: RNA interference: Oligonucleotide duplexes were transfected with Lipofectamine 2000 into cells. Centrosome preparation: HeLa cells were pelleted, washed, lysed, and subjected to centrifugation with a 60% sucrose and a discontinuous gradient centrifugation consisting of 70%, 50% and 40% sucrose solution. Immunofluorescence: Cells grown on cover slips were fixed with 4% paraformaldehyde or methanol. Following extensive washing, cells were blocked with 3% BSA, incubated with primary antibodies overnight at 4°C in 3% BSA and then detected with Alexa-conjugated fluorophores. Results: Centrosome abnormal amplification is one of the major reasons of mitotic defect and chromosome instability in cancer cells. Several of DNA/RNA binding proteins are found to regulate centrosome duplication including geminin, nucleophosmin(NPM) and p53. Here, we find Sam68, a splicing factor, is involved in centrosome duplication and its localization on centrosome in mitosis is regulated by Ran-Crm1. First, we find depletion of Sam68 by using RNA interfering causes centrosome overduplication in both human and mouse cell lines. Centrosome aberration also induces G2/M block and mitotic failure. Second, we use several approaches to distinguish between centrosome overduplication and centrosome accumulation by cell division failure. It shows that loss of Sam68 induces centrosome duplication not cell division failure. At last, we also find Sam68 and NPM major localize on centrosome in M and G1 phase, and their localization is coordinated with geminin which major localize on centrosome in G1/S and S phase. It is also very interesting that by treatment with leptomycin B, an inhibitor of Crm1, Sam68 is lost from centrosome.
treatment induces premature centrosome duplication in quiescent cells, which coincides with Sam68 and NPM dissociation from centrosomes. The Ran-Crm1 complex may promote a local enrichment of Sam68 on centrosomes, thereby preventing centrosome reduplication. Conclusion These results indicate that Sam68 might be an important factor that participates in ensuring the fidelity of centrosome duplication once per cell cycle.

1846/B225

The License for Centriole Duplication.

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The centrosome duplicates once per cell cycle in most dividing cells. The duplication process involves the alternating assembly and disengagement of centriole pairs at the core of each centrosome. Based on studies with Xenopus egg extracts, it has been proposed that separase-dependent centriole disengagement at anaphase licenses centrosomes for duplication in the next cell cycle. Here we use genetics and chemical biology to test whether such a mechanism exists in intact human cells. Homozygous deletion of the locus encoding separase caused a strong block to centriole disengagement during mitotic exit, which in turn inhibited timely assembly of new centrioles during the following S phase. However, most engagements were eventually dissolved, implying the existence of an additional activity that destabilizes centriole engagement. Using specific pharmacological and chemical genetic inhibitors, we identified Polo-like kinase 1 (Plk1) as an essential activator of centriole disengagement. Timed addition of these inhibitors mapped Plk1’s execution point vis-à-vis disengagement to late G2 or early M phase, i.e., temporally upstream of securin destruction and separase activation at anaphase onset. Crucially, when cells exited mitosis after downregulation of both separase and Plk1, centriole disengagement failed completely, and subsequent centriole duplication in interphase was also blocked. Our results indicate that the catalytic activities of Plk1 and separase act throughout M phase to license centrosome duplication, reminiscent of their roles in removing cohesin from chromosomes.

1847/B226

Centrosome Separation at G2/M Licenses Centrosome Duplication in a Cdk1/Eg5-dependent Manner.

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To study the mechanism of centrosome duplication in cycling cells, we established a novel system of multiple centrosome formation in two types of cells: CHO cells treated with a cdk1 inhibitor (RO3306) and DT40 cells, in which one of the functionally redundant interphase cdk5 (cdk1 and cdk2) was inactivated (Hochegger et al., 2007). Cdk-inhibited cells initiated DNA replication, which triggered centrosome duplication at the onset of S phase. Although these cells became arrested at G2/M, the centrosome cycle continued to produce supernumerary centrioles/centrosomes in these cells. The centrosomes were consisted of centrioles that generally were not in the typical orthogonal configuration. The expression of p53 and geminin inhibited both DNA replication and centrosome duplication. The G2/M-arrested cells did not endoreplicate DNA, but instead continued to grow, and their cellular mass increased over 10-fold during 48 hr culture of DT40 cells. When cdk1 and cdk2 were simultaneously blocked, the centrosome cycle ceased but the cells continued to grow, indicating that three cycles (DNA replication, centrosome duplication, and cell growth) are coupled in a cdk-dependent manner. While no centrosomes were assembled, molecules necessary for the construction of new centrosomes appeared to be synthesized and accumulated in double knockout cells. Multiple centrosomes were adjacent to the nuclear membrane and widely separated from each other. One of the molecules responsible for centrosome separation is Eg5, a mitotic kinesin-like motor
protein, which is a cdk1 target. When cells were treated with Eg5 inhibitors, separation and multiplication of centrosomes did not occur in either CHO or DT40 cells. These results suggest that centrosome separation at the onset of M phase is involved in the licensing mechanism of centrosome duplication.

1848/B227
Phosphoproteome Analysis and Phospho-regulation of the Yeast Centrosome.
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The yeast centrosome (known as the spindle pole body, SPB) nucleates microtubules in the nucleus to form the mitotic and meiotic spindles. Much is known about the structure and function of the SPB; however, the regulation of SPB duplication and function is yet to be well described. Protein phosphorylation is a ubiquitous mechanism for cellular regulation and signaling. Prior to this study, only a few phosphorylation sites had been identified on SPB proteins, some of which contribute to assembly and function of the SPB. Our goal is to provide a comprehensive inventory of phosphorylation events found on all 18 core SPB proteins from intact SPB complexes using mass spectrometry. This information will be used to uncover regulatory mechanisms of SPB assembly and function, and to assign known regulators, such as Cdc28 and Mps1 kinase, to particular phosphorylation events. We have now identified 274 novel phosphorylation sites on SPBs isolated from asynchronously growing and cell cycle arrested (G1 and mitotic) yeast cultures. The cell cycle arrested samples are of particular importance, as they can correlate timing of phosphorylation events with specific functions. We will prioritize the analysis of sites according to the degree of regional and local conservation of the phosphorylated amino acid, and also whether the phosphorylation state changes during the cell cycle. There are many studies on orthologous proteins which have demonstrated the importance of a conserved phosphorylated amino acid on protein function. We have identified numerous conserved phosphorylation sites across many of the 18 SPB proteins, and we are currently exploring the effect of mutating several such sites within the gamma tubulin complex. Overall, this project will greatly expand our understanding of centrosome regulation and contribute a wealth of knowledge to the community for future studies on phospho-regulation.

1849/B228
Phosphorylation-dependent Regulation of Fbxw7/hCdc4 Stability by Polo-like Kinase-2 Controls Cyclin E Protein Levels.
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Polo-like kinases (Plks) perform crucial functions in cell-cycle progression and during mitosis. Mammalian cells express four Polo-like kinase family members - Plk1, Plk2(Snk), Plk3(Fnk,Prk) and Plk4(Sak). Plk2 is activated near the G1/S phase transition and is involved in the reproduction of centrosomes. The kinase is localized to centrioles during early G1 phase where it only associates to the mother centriole and then distributes equally to both mother and daughter centrioles at the onset of S phase. In addition, we found that Plk2 cooperates with both Cdk2 and Plk4 in regulating centriole duplication. However, the mechanism underlying Plk2-induced centriole duplication is still unknown. Here, we show that Plk2 co-operates with Cdk2/Cyclin E. Plk2 is involved in the regulation of Cyclin E protein stability since RNAi-mediated down-regulation of Plk2 results in a decrease of Cyclin E. This leads to a diminution of phosphorylation levels of centrosomal Cdk2/Cyclin E substrates, namely NPM B23 and CP110. Furthermore, we find that Plk2 directly targets the F-box protein SCF Fbxw7 which is involved in ubiquitin-mediated degradation of Cyclin E. Phosphorylation of Fbxw7 by Plk2 at three conserved serine residues, Ser25, Ser176 and Ser349 induces destabilization of the F-box protein by ubiquitin-mediated degradation resulting in accumulation of Cyclin E. Our results define a novel Plk2 dependent pathway regulating the onset of S-phase and centrosome duplication.
1850/B229

**Polo-like Kinase 4 Regulates Its Own Stability.**
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Centrosomes are the major microtubule-organizing centers of animal cells and are comprised of a pair of centrioles surrounded by an amorphous pericentriolar material. Centrosomes play an important role during mitosis, where they form the poles of the bipolar microtubule spindle upon which chromosomes are segregated. Extra centrosomes can cause errors in spindle formation that lead to subsequent chromosome missegregation and thus, accurate control of centrosome number is critical for the maintenance of genomic integrity. In vertebrates and invertebrates, the protein kinase Polo-like kinase 4 (Plk4) plays an important role in initiating centriole duplication and overexpression of Plk4 promotes centriole overduplication and the subsequent formation of supernumerary centrosomes. Here we show that active Plk4 is less stable than a kinase dead mutant and is specifically targeted for degradation by the proteasome. Mass spectrometry identified a 24 amino acid rich region that is multiply phosphorylated by Plk4 in vitro. Deletion of this region prevented kinase active Plk4 from promoting its own destruction. Phosphorylation of multiple sites in this region was required to degrade Plk4, indicating a threshold level of kinase activity is required for the proteins destruction. We propose that Plk4 kinase activity autoregulates the proteins stability and that this is an important mechanism to self-limit kinase activity and prevent the disastrous consequences of centriole overduplication.

1851/B230

**A Role of Aurora-A and NDEL1 during Neurite Outgrowth in DRG Neurons.**
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Orchestrated remodeling of cytoskeleton is prominent during neurite extension. Although actin filament regulation has been extensively characterized during neurite extension, it remains largely unknown how the dynamics of microtubules and MTOC (microtubule organizing center) are regulated. Among the MTOC-related proteins, we have been focusing on the molecular interaction between Aurora-A and NDEL1, both of which are important during the cell cycle, especially to overcome the check point of G2/M phase. Activated and phosphorylated Aurora-A sequentially binds and phosphorylates NDEL1 at the centrosome from later G2 phase to promphase, which is required for centrosome maturation, separation and the microtubule organization. Given that NDEL1 is essential for dynein complex transport and neurite elongation with Lis1 and DISC1, we here elucidated the molecular mechanism how NDEL1 is activated in neuron. We first examined the expression of Aurora-A in the DRG neuron, and found that Aurora-A and NDEL1 were co-expressed in the centrosome or MTOC at the neurite hillock. Furthermore, we found that the atypical protein kinase C (aPKC)-Aurora-A-NDEL1 pathway plays a critical role in the regulation of microtubule organization during DRG (dorsal root ganglia) neurite extension. Our biochemical and cell biological analysis revealed that aPKC phosphorylates Aurora-A at T287, which augments interaction with TPX2 and facilitates activation of Aurora-A at the DRG neurite hillock, followed by S251 phosphorylation of NDEL1 and its recruitment. Suppression of aPKC, Aurora-A, or disruption of NDEL1 resulted in severe impairment of neurite extension. Analysis of microtubule dynamics using microtubule plus-end marker revealed that suppression of the aPKC-Aurora-A-NDEL1 pathway reduces the frequency of microtubule emanation from the MTOC, suggesting that Aurora-A is downstream of aPKC for the regulation of microtubule dynamics. These findings indicate the pivotal role of the aPKC-Aurora-A-NDEL1 pathway in microtubule remodeling during neurite extension.

1852/B231

**The Cilia Protein IFT88 Forms Novel Mitotic Complexes and Functions in Spindle Poles Organization and Spindle Orientation.**
Centrosomes contribute to spindle organization/orientation in mitosis and mediate primary cilia assembly in noncycling cells. Ciliogenesis is accomplished through intraflagellar transport (IFT), a bi-directional motility system in the cilium that requires multimeric protein complexes called IFT particles and motors such as kinesins or cytoplasmic dynein2 to transport cilia components. IFT proteins and other cilia proteins contribute to ciliopathies when disrupted. Some cilia proteins, including IFT proteins, also localize to centrosomes/spindle poles in mitotic cells, but their role in cell division is largely unexplored. Here, we test the role of IFT proteins in mitosis. Depletion of IFT88 from zebrafish embryos and cultured human cells induced profound defects in mitotic spindle organization/function. Most prominent were decreased microtubule nucleation and defocused mitotic spindle poles. Consistent with these phenotypes was a reduction in the microtubule nucleating protein γ-tubulin and astral microtubules at spindle poles. Consequently, astral-microtubule-dependent orientation of the mitotic spindle and the plane of cell division were disrupted. To understand the molecular basis for IFT88 function in mitosis, we searched for IFT88 interacting partners. EB1 and cytoplasmic dynein1 immunoprecipitated with IFT88 in mitotic cell lysates. IFT88 depletion caused selective and dramatic loss of pole, but not spindle, localization of EB1. Depletion of dynein1, but not IFT-associated dynein2, disrupted IFT88 pole localization and caused its redistribution to a pericentrosomal region. In conclusion, we identified unanticipated mitotic complexes and functions for IFT88 at spindle poles. We propose that IFT88 is part of a previously uncharacterized microtubule-based dynein1-driven cargo transport system in mitosis analogous to the cilia counterpart. This complex could anchor/transport proteins such as EB1 and γ-tubulin to spindle poles where they would perform their established roles in astral microtubule nucleation and organization. Our work provides new insights into the mechanism of misorientation of the mitotic spindle and cell division plane, both potential contributors to cystogenesis and ciliopathies.

1853/B232
SAK/PLK4 Regulation of Protein Levels by the SCF/Slimb Complex throughout the Cell Cycle.

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Centrioles are essential for the formation of microtubule-derived structures, such as cilia and centrosomes. Abnormalities in centrosome number and structure occur in many cancers and are associated with genomic instability. In most dividing animal cells, centriole formation is coordinated with DNA replication and is highly regulated such that a single daughter centriole forms close to each mother centriole per cell cycle. Centriole formation is triggered and dependent on a conserved kinase, SAK/PLK4. Both downregulation and overexpression of SAK/PLK4 are associated with cancer in humans, mice, and flies. We shown how centrosome amplification is normally inhibited by degradation of SAK/PLK4, which is mediated by the SCF/Slimb ubiquitin ligase. This complex physically interacts with SAK/PLK4, and in its absence, SAK/PLK4 accumulates, inducing the formation of multiple daughter centrioles surrounding each mother. This interaction is mediated via a conserved Slimb binding motif in SAK/PLK4, mutations of which leads to centrosome amplification. Because centriole duplication is a cell cycle controlled mechanism, it is of utmost importance to unravel the timing and regulation of this regulation. To this end we are currently characterizing the regulation of SAK/PLK4 degradation using a phosphospecific antibody that recognises SAK/PLK4 phosphodegron. Candidate kinases to regulate SAK/PLK4 levels are being screened by RNAi using this tool.
1854/B233
An Essential Mechanism Dependent on Ase 1/Prc1 Ensures Merotelic Correction During Anaphase In Fission Yeast.
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Faithful segregation of sister chromatids requires the attachment of each kinetochore to microtubules that extend from opposite spindle poles. Merotelic kinetochore orientation is a kinetochore -microtubule mis-attachment in which a single kinetochore binds microtubules to both spindle poles rather than just one. We genetically induced merotelic kinetochore attachment in fission yeast during anaphase and found that it leads to intra-kinetochore stretching followed by either correction or kinetochore disruption. Laser ablation of spindle microtubules reveal that intra-kinetochore stretching and merotelic correction is dependent on microtubule forces. We find that the presence of multiple merotelic chromosomes linearly antagonizes the spindle elongation rate and that this phenomenon can be solved numerically using a simple force balance model. Based on the predictions of our mechanical model, we provide In Vivo evidence that correction of merotelic attachment in anaphase is tension-dependent and requires an Ase1/Prc1-dependent mechanism that prevents spindle collapse and hence by asymmetric division or the appearance of the cut phenotype.

1855/B234
Signaling Mechanisms That Regulate Kinesin-mediated Mitochondrial Motility.
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Mitochondria are mobile organelles and cells regulate mitochondrial movement in order to meet the changing energy needs of each cellular region. Elevated local Ca2+, a likely stress signal, halts both anterograde and retrograde mitochondrial motion. A kinesin/adaptor complex which contains milton and a Ca2+-binding protein, Miro, mediate this effect. We show that kinesin is present on all axonal mitochondria, including those that are stationary or moving retrograde, and this association is not disrupted by high Ca2+. Instead, Ca2+-binding permits Miro to interact directly with the motor domain of kinesin-1, preventing motor/microtubule interactions. Thus, kinesin-1 switches from an active state in which it is bound to Miro via milton, to an inactive state in which direct binding to Miro prevents its interaction with microtubules. Furthermore, this mechanism explains the arrest of mitochondrial motility in dendrites by the activation of glutamate receptors and thereby enhances the resistance of neurons to excito toxicity. We also show that the glucose sensor O-GlcNAc Transferase (OGT) and a kinase mutated in Parkinsonism, PINK1, both of which interact with this kinesin/adaptor complex, arrest mitochondrial motility. Glycosylation and phosphorylation may therefore represent additional regulatory inputs to this complex. These signaling mechanisms may allow mitochondria to alter their distribution in response to local conditions in subcellular domains.

1856/B235
Quantitative Analysis of Actin-Mediated Chromosome Congression in Starfish Oocytes.
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A dynamic F-actin network in the nuclear region of starfish oocytes is essential during meiosis for transport of chromosomes to within capture distance of astral microtubules. Here, we apply quantitative image analysis and computational modeling to investigate the molecular mechanism of the capture and directed transport of chromosomes by this network. Image-based fluorescence correlation procedures are used to quantify spatiotemporally-dependent rates of F-actin
assembly, disassembly, and transport globally at the nuclear-level, as well as locally at individual chromosomes. Particle-tracking procedures are used to quantify distinct regimes of chromosome transport during the congression process. We find three distinct phases of chromosome motion: random walks prior to nuclear envelope breakdown that transition to more directional but lower average velocity motion upon capture by the actin network, followed by high velocity directional motion upon microtubule capture. Analyzing the actin-dependent phase in more detail reveals that the local velocity of both the actin network and the chromosomes increases linearly with increasing distance from the spindle pole, consistent with a model of congression in which chromosomes are embedded in a uniformly-contracting actin network. Finally, we use image-based unsupervised learning techniques to discover characteristic structural features of this F-actin network, as well as to compare it quantitatively with a conserved dynamic actin network that mediates spindle transport in mouse oocytes. The quantitative hypotheses of chromosome capture and transport provided by this integrative computational approach will be tested experimentally using direct molecular perturbation.

1857/B236
**Bves Interacts with VAMP3 to Regulate Vesicular Transport.**
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Blood vessel/epicardial substance (Bves) is a transmembrane protein that influences cell adhesion and motility through unknown mechanisms. We have discovered that Bves directly interacts with VAMP3, a SNARE protein that facilitates vesicular transport and specifically recycles transferrin and β1 integrin. Two independent assays document that cells expressing a mutated form of Bves are severely impaired in the recycling of these molecules, a phenotype consistent with disruption of VAMP3 function. Using a Morpholino knockdown and rescue system in Xenopus laevis, we demonstrate that elimination of Bves function specifically inhibits transferrin receptor recycling and results in gastrulation defects that suggest impaired cell movement. Time-lapse analysis of isolated Bves-depleted cells In Vitro revealed severe impairment of cell spreading on fibronectin, indicative of disruption of integrin-mediated adhesion. Taken together these data demonstrate that Bves interacts with VAMP3 and facilitates receptor recycling both In Vitro and during early development. Thus, this study establishes a newly identified role for Bves in regulation of vesicular transport and reveals a novel, broadly applied mechanism governing SNARE protein function.

1858/B237
**Microtubule-dependent Transport and Cell Surface Exposure of MT1-MMP in Macrophages Is Powered by KIF5B and KIF3A/KIF3B Kinesins and Rab8.**
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Matrix metalloproteinases (MMP) play key roles in tumor cell invasion, but also in matrix degradation by non-transformed somatic cells. Here, we investigate the role of membrane-bound MT1-MMP in primary human macrophages, in particular the molecular mechanisms regulating intracellular trafficking, exposure on the cell surface, and podosome-dependent matrix degradation. We find that MT1-MMP is transported in vesicles from a Golgi/recycling endosome compartment in the cell interior to the cell periphery. for long-range transport, these vesicles move along microtubules, but can also attach to microfilaments in the cell periphery. SiRNA-mediated knock down shows that anterograde transport along microtubules is driven by the KIF5B and heterotrimeric KIF3A/KIF3B kinesins, while retrograde transport depends on dynein, as determined by dynamitin overexpression. In line with recent reports, we also find that the GTPase Rab8 is critical for MT1-MMP recycling. Consistently, use of dominant negative constructs of KIF5B, KIF3A/KIF3B and Rab8, as well as siRNA-mediated knock down of KIF5B and KIF3A/KIF3B, significantly reduces surface exposition of MT1-MMP, as well as gelatin degradation by macrophages. We are currently studying other Rab isoforms to further determine
the trafficking pathways that mediate exposure of MT1-MMP on the macrophage cell surface, as well as podosome-localized matrix degradation by these cells.

1859/B238

**Collective Motility of Structurally-Defined, Macromolecular Complexes of Molecular Motors.**

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Many intracellular transport processes rely on the collective function of multiple motor proteins. Yet, how groups of motors function is not understood, particularly since the exact number and type of motors coupled to a cargo particle is unknown in most experiments. Objective: Our objective is to create structurally-defined multi-motor complexes so that collective motor mechanics can be investigated with the same levels of control and precision available to single-motor biophysical assays. Methods: Our methods facilitate studies of collective motor function both In Vitro and in living cells. To this end, we have synthesized multiple motor assemblies composed of two-coupled kinesin-1 motors. Assemblies are formed using a single duplex of DNA as a molecular scaffold and DNA-conjugated artificial polypeptides as capture probes that form stable intermediate linkages between motor proteins and the scaffold. Complexes containing multiple dynein motor have also been created using specific capture molecules. In this case, engineered cargos are outfitted site-specifically with multiple dyneins inside living cells. Results: We have discovered a previously uncharacterized collective transport phenomenon: when multiple kinesins function collectively they impose forces on one another transiently. Tension developed within a multiple motor systems increases motor-microtubule detachment rates. Furthermore, we have developed new techniques that facilitate the creation of engineered intracellular cargos that target to dynein-dependent transport pathways. Conclusion: Our results reveal that the communication of forces between motors dominates collective motor mechanics. This type of intra-motor coupling leads to inhibited motor function at low applied loads, but can yield enhanced motor activity at high loads. Nevertheless, the cytosol can impose conditions on motor transport that are not recapitulated during In Vitro assays. Consequently, the construction of engineered intracellular cargos with defined compositions will be essential to resolving mechanisms of collective motor transport in living cells.

1860/B239

**On a Mechanism of Cytolytic Granules Trafficking in Cytotoxic T Lymphocytes.**

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Cytotoxic T lymphocytes (CTL) kill target cells by delivering cytolytic granules at the CTL/target cell interface. We analyzed pattern of granule polarization and kinetics of their release by CTL and found that these parameters are regulated by the kinetics of TCR early signaling that determine increase in the level of intracellular Ca2+. These data lead us to propose a model according to which the granules can travel to the secretory domain via two different pathways. The short pathway constitutes swift granule recruitment to the MTOC and subsequent delivery by the polarized MTOC directly to the secretory domain. The longer path is characterized by late recruitment of the granules that move along microtubules to the periphery of the synapse and then move tangentially to fuse at the outer edge of the secretory domain. The mechanism regulating the balance between the two paths will be discussed.

1861/B240

**Rab Conversion Allows Melanosome Track Switching from Microtubule- to Actin-Based Transport.**
Background. Previous studies reported Rab32/38 regulates biogenesis of immature melanosomes by controlling Tyrp1 trafficking. Meanwhile Rab27a, plus effectors Melanophilin (Mlph) and MyosinVa (MyoVa), is proposed to retain mature melanosomes in peripheral melanocyte dendrites by switching them from microtubule (MT) to actin based transport. Objective. The aim of this study was to directly investigate the relationship between cytoskeleton track switching and Rab27a and Rab32/38 recruitment in living cells. Methods. To address this issue we transiently expressed EGFP and mRFP tagged Rab and Mlph proteins in melanocytes and used time-lapse confocal microscopy to record the movement of melanosomes and associated proteins in the presence and absence of cytoskeleton disrupting drugs. We then used Volocity software to automatically track the movements of melanosomes thereby allowing us to correlate movement characteristics with melanosomal Rab content. Results. Single transfection experiments show Rab27a recruitment switches melanosomes from MT to actin dependent transport. This suggests that Rab27a mediates track switching by recruiting downstream effectors Mlph and MyoVa. Consistent with this we found melanosomal Mlph recruitment in wild-type and Mlph null cells correlated with switching from MT to actin dependent transport while Rab27a recruitment to melanosomes in Mlph null cells did not. Rab27a and Mlph double transfection also confirmed that association of the two proteins correlates with track switching. Analysis of Rab32/38 expressing cells revealed that these proteins preferentially associate with fast moving melanosomes consistent with their role in trafficking to immature melanosomes. Finally analysis of movement in Rab27a and Rab32/38 double transfected cells revealed that the content of these Rabs is inversely related, with fast moving melanosomes containing high levels of Rab32/38 and low levels of Rab27a and vice versa for slow moving melanosomes. Conclusions. These data provide direct evidence that Rab27a and effectors Mlph and MyoVa mediates track switching of melanosomes from MT to actin and suggest that melanosomal Rab conversion may integrate organelle maturation and transport processes.


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Lacrimal gland (LG) acinar cells are specialized epithelial secretory cells responsible for production and release of tear proteins. The purpose of this study was to characterize the effect of Rab27a and Rab27b loss in the LG, in order to better understand the role of Rab27 In Vivo in these cells. Methods: LG pairs from male ashen/Rab27b-/- double-knockout (DKO) and parent C57BL/6 (WT) mice (n=5 of each) were fixed for EM sectioning and cryo-IF and histology analysis. EM images were analyzed with Image J to measure individual secretory vesicle (SV) size and distance from the lumen and to count specific organelles in each cell. Results: DKO LG acinar cells demonstrated a general loss of cellular organization and morphological changes in multiple organelles. Greater vesiculation of the ER was evident in DKO versus WT cells. A decrease in SV count (96±15 per cell in WT versus 52±7 per cell DKO) and a decrease in average SV size (0.835±0.087μm WT versus 0.810±0.091μm DKO) was detected. SVs were more widely scattered in DKO acinar cells; the average distance from the lumen increased from 32±2% to 38±2%. A 2.2-fold increase in lysosomes was also supported by observations of increased LAMP-2 staining in DKO LG frozen sections. While total mitochondria did not change, DKO acinar cells displayed an increase in abnormally vesiculated or swollen mitochondria. Conclusions: Rab27 isoforms are known to participate in intracellular trafficking of SV in various cells. Consistent with our previous In Vitro studies in rabbit LG acinar cells, we find that DKO
mice express morphological changes in SV reflecting a regulatory role for Rab27 
in LG SV formation and maturation. This study, however, also suggests that the DKO effect is more widespread than in the secretory pathway alone. Loss of both Rab27 isoforms not only changed SV expression, but also affected the morphology of other organelles. Disruption of the secretory pathway in these specialized epithelial cells may affect the general health of the cells through generation of metabolic stress due to inability of newly synthesized proteins to be appropriately packaged into the secretory machinery. [Support: NIH EY011386; EY10550]

1863/B242
Robust Transport by Multiple Motors with Non-linear Force-Velocity Relations and Stochastic Load Sharing
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Transport by processive molecular motors (Kinexin, Dynein and Uncoventional Myosin) plays an important role in many cell biological phenomena. In many cases, motors work together to transport cargos in the cell, so it is important to understand mechanics of the multiple motors. Based on earlier modeling efforts, we have studied effects of nonlinear force-velocity relations and stochastic load sharing on multiple motor transport. We find that when two or three motors transport the cargo, then the nonlinear and stochastic effects compensate so that the mechanical properties of the transport are robust. Similarly, the transport is insensitive to compliance of the cargo-motor links. Furthermore, the rate of movement against moderate loads is not improved by increasing the small number of motors. When the motor number is greater than three, correlations between the motors become negligible, and the earlier analytical mean-field theory of the multiple motor transport holds. In the limit of the great number of coupled motors, we demonstrate how mechanics of the multiple motors scale with the motor number.

1864/B243
Signaling Unit Composed of PP2A and CK1 Is Responsible for a Rapid Increase in the Minus-End Runs of Pigment Granules Along Microtubules during Pigment Aggregation in Melanophores.
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Bidirectional transport of organelles and particles along the cytoplasmic microtubules (MTs) involves microtubule motors, kinesins and dyneins. While it is known that MT transport direction is tightly regulated in living cells, the mechanisms of this regulation remain poorly understood. In Xenopus melanophores, direction of transport of pigment granules is regulated by the levels of second messenger, cAMP, and involves the activities of Protein Kinase a (PKA) that induces pigment transport to the MT plus ends located at the cell periphery (dispersion), and Protein Phosphatase 2A (PP2A) required for the movement of pigment granules to MT minus ends clustered in the cell center (aggregation). A rapid onset of pigment aggregation suggests, however, that other signaling molecules operate downstream of PP2A to amplify the signal that leads to enhance the length of minus-end MT runs of pigment granules. Here we tested the possibility that signaling during pigment aggregation involves Casein Kinase 1 (CK1), which has been shown to transmit signals downstream of PP2A to amplify the signal that leads to enhance the length of minus-end MT runs of pigment granules. We found that one of the CK1 isoforms, CK1ε, copurified with pigment granules, and that GFP-tagged CK1ε was bound to pigment granules in cells. The CK1ε-specific inhibitor IC261 prevented pigment aggregation in the cells with dispersed pigment granules, and induced pigment dispersion in the aggregated cells. The measurements of CK1 and PP2A activities in the preparations of pigment granules isolated from the cells with aggregated or dispersed pigment indicated that activities of both enzymes significantly increased in response to pigment aggregation stimuli. Remarkably, a CK1 inhibitor (IC261) partially
prevented an increase in the PP2A activity, whereas a PP2A inhibitor (okadac acid) reduced the CK1 activity increase seen during aggregation. Our data are consistent with a model for pigment aggregation signaling in which CK1 and PP2A stimulate each other activities, and this mutual stimulation amplifies the pigment aggregation signal, which ensures a rapid increase in the dynein-dependent runs of pigment granules to the MT minus ends.

1865/B244
Regulation of Microtubule Dynamics Enhances Capture of Pigment Granules by Growing Microtubule Ends During Pigment Aggregation in Melanophores.

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Cytoplasmic microtubules (MTs) continuously search the intracellular space and make contacts with various targets by means of dynamic instability, growth and shortening at the plus-ends. We have recently found that dynamic instability is required for initiation of the minus-end directed transport of pigment granules in Xenopus melanophores. The major function of these cells is fast and synchronous redistribution of thousands of pigment granules, which aggregate in the cell center or uniformly disperse throughout the cytoplasm. During aggregation, pigment granules moving along the actin filaments must transfer onto MTs for dynein-dependent transport to the MT minus ends clustered in the cell center. We have shown that this transfer involves capture of pigment granules by the growing MT plus-ends enriched in the plus-end tracking protein CLIP-170. Here we used live cell imaging of melanophores expressing GFP-CLIP-170 to determine whether aggregation signals facilitate binding of pigment granules to MTs through an increase in the levels of the plus-end bound CLIP-170, and/or the amount of the CLIP-170-enriched MT plus-ends. We found that in the cells stimulated to aggregate pigment granules the average length of GFP-CLIP-170 comets and the number of MT ends enriched in GFP-CLIP-170 raised by ~20% and ~100%, respectively. To estimate the effects of these changes on the global kinetics of pigment aggregation we used a spatial stochastic computational model for pigment transport in melanophores. Kinetic Monte-Carlo simulations indicated that an increase in the number but not the length of GFP-CLIP-170 comets led to a substantial (~2-fold) reduction in the pigment aggregation half-time. We conclude that in melanophores aggregation signals enhance capture of pigment granules by MTs by increasing the amount of MT plus ends enriched in the CLIP-170.

1866/B245
Tug-of-war between Dissimilar Teams of Motors: Key to Regulated Bidirectional Transport and Fission of Endosomes.

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We extend single-molecule-like optical trap measurements to bidirectional (back-and-forth) motion of endosomes on microtubules. Experiments at three levels of complexity - inside cells, with cell extract and motor-coated beads, demonstrate that reversals during motion arise from tug-of-war between plus-directed kinesin and minus-directed dynein motors. We precisely measure three defining parameters of this tug-of-war: Force of single/multiple motors, numbers of opposing motors and motor’s response to applied load. Surprisingly, 5-7 weak detachment-prone dyneins on the endosome membrane engage in tug-of-war against 1 strong and tenacious kinesin. This unique choice of dissimilar motor-teams achieves net minus transport together with endosome fission, both likely important in controlling the balance in endocytic sorting. To the best of our knowledge, this is the first demonstration of an In Vivo process that requires and utilizes the difference in molecular properties of kinesin and dynein. Our work may provide a platform to understand intracellular transport of a variety of organelles in terms of measurable quantities.
Macrophages are major effector cells of the innate immune response, and are specialized to recognize and respond to pathogens. To carry out their function, macrophages must adeptly migrate from blood to target tissues. Endothelial transmigration is accomplished by matrix metalloproteinase (MMP)-induced degradation of basement membrane and ECM components. Activation of macrophages with LPS and IFN-γ causes increased cell spreading, enhanced phagocytosis and increased secretion of MMPs, particularly MMP-9. Although the role of MMP-9 in macrophage migration has been characterized, the intracellular means of trafficking and secretion of MMP-9 remain unclear. We show by gelatin zymography and western analysis that MMP-9 secretion commences 6 hours after activation, with greatest intracellular levels obtained at 9 hours. Immunofluorescent imaging of macrophage cells activated for 9 hours revealed MMP-9 vesicular structures that colocalized with the microtubule (MT) cytoskeleton. Cells treated with nocodazole to disrupt the MT cytoskeleton lacked the ability to secrete MMP-9 to the cell exterior. Additionally, nocodazole treated cells showed a significant build up of MMP-9 intracellularly. Based on these results and on previous work in our lab showing an increase in stable cytoplasmic MTs after activation of macrophage cells, we suggest that stabilized MTs may function as highways to mediate the extensive targeting of MMP-9 to the cell surface in activated macrophages.

Differences in Biophysical Properties Underlie the Limited Regenerative Ability of Adult as Compared to Embryonic Peripheral Neurons.

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The intrinsic differences between adult and embryonic neurons that limit axonal regeneration following injury are not well understood. Recent work suggests that axonal elongation occurs through a two step process where forces at the growth cone stretch the axon and new material is added along the axonal shaft. Our mathematical modeling predicts the rate of axonal stretching / elongation is a function of the level of force generation at the growth cone, the strength of adhesions of the axon to the substrate, axonal diameter, and the mechanical stiffness (i.e. viscosity) of the axon. To test the hypothesis that biophysical differences that underlie the limited regeneration of adult dorsal root ganglion (DRG) neurons, we used force calibrated towing needles to compare their properties. We found the axons of adult DRG neurons had both stronger adhesions and a higher viscosity than the axons of embryonic neurons. Our results suggest decreasing axonal stiffness and /or adhesion to the substrate of adult axons could accelerate their regeneration.

Imaging APP and JIP1/2 in Action: Quantitative Spatiotemporal Dynamics of Transport in Squid Giant Axons.

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Transport of membrane-bound cargo in neurons is heterogeneous both in its molecular detail, and its spatiotemporal dynamics. By injecting 100nm peptide-conjugated fluorescent beads into the squid giant axon, we identified a 15-aa peptide from APP that mediates fast axonal transport of beads. JIP1/2, a scaffolding protein implicated in transport, binds both to the TRP motif of kinesin light chain and the GYENPTY motif of APP. Here we test whether the kinesin-binding domain of JIP (aa 698-711) can hitch cargo to motors for transport. We further perform competition experiments by co-injecting negative-charge (carboxylated) beads with APP- and/or
JIP-beads, or APP with JIP into the same axon. Counts of motile vs. stationary beads in time-lapse videos revealed that a large fraction of carboxylated, APP- or JIP-beads exhibit anterograde motion (92.8±1.7%, n=413; 88.4±1.3%, n=433; 89.8±2.3%, n=357 respectively) whereas glycine conjugation abolished this capacity. When co-injected with glycine beads, carboxylated beads sustained motility, but when co-injected with either APP- or JIP-beads, carboxylated beads gradually stalled while peptide-beads sustained motility. Conversely fewer APP-beads moved when co-injected with JIP-beads. Similarly, co-injection of soluble peptides with beads demonstrated that JIP is up-stream of APP: APP peptide impaired APP-bead motility (18.2±0.5% motile), but had less effect on JIP-beads (53.9±8.3% motile), while soluble JIP peptide decreased both JIP- and APP-bead motility (JIP, 19.25±3.7% motile; APP, 17.89±7.1% motile). Average instantaneous velocities (APP, 0.41±0.07µm/s; JIP1/2, 0.46±0.17µm/s) and track lengths (7.9±3.58µm) indicate that bead movement replicated physiological transport. Thus APP and JIP both collaborate and compete for motors. In peptide-bead pull-downs APP primarily interacted with kinesin-1, whereas JIP preferentially pulled down kinesin-3 and much less kinesin-1. Our results show that APP and JIP are capable of recruiting more than one kinesin with different affinities. Thus, elucidating the minimal peptide “zipcode” for motor recruitment reveals biological strategies that allow fine control of transport dynamics. Supported by NINDS NS046810.

1870/B249
Quantitative Analysis of GLUT4 Storage Compartment Formation in 3T3-L1 Adipocytes with Quantum Dot-based Single Molecular Imaging.
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Glucose transporter 4 (GLUT4) is among the most important proteins for insulin action in adipose and muscle cells. To quantitatively analyze GLUT4 dynamics, we previously developed a novel approach based on single molecular imaging using the quantum dot (Qdot). With this approach, using 3T3-L1 adipocytes, we have described insulin action quantitatively, and obtained evidence indicating both the release of GLUT4 from storage compartment and its prerequisite formation to be important for appropriate insulin action. In this study, employing the Qdot-based approach, we quantitatively investigated the mechanism of GLUT4 storage compartment formation in 3T3-L1 cells. We labeled exofacial myc-tagged GLUT4-ECFP with Qdot-labeled anti-myc antibody in 3T3-L1 cells. We tracked the trajectories of each Qdot fluorescence and analyzed molecular dynamics in terms of movement speed and mean-square displacement. First, we found the intracellular motility of GLUT4 to differ between undifferentiated fibroblasts and differentiated adipocytes (fibroblast speed, 0.86±0.34 µm/s; adipocyte speed, 0.31±0.03 µm/s; P<0.001). The movement of GLUT4 decreased as differentiation progressed, and on differentiation day 4, motility features were similar to those of fully-differentiated adipocytes. We also found the motility of transferrin receptor (tracked by Qdot-transferrin) in adipocytes to differ from that of GLUT4, whereas in fibroblasts the two were quite similar, providing direct evidence that a specialized anchor mechanism(s) for GLUT4 may exist only in differentiated 3T3-L1 adipocytes. To identify factors possibly involved in this anchor mechanism(s), we looked for a protein which decreases GLUT4 movement in 3T3-L1 fibroblasts, and identified a candidate; sortilin, a type I transmembrane receptor, expression of which is induced from day 3-4 of differentiation. Overexpression of sortilin reduced the motility of GLUT4, but not that of transferrin receptor, in 3T3-L1 fibroblasts. Thus, we directly observed the GLUT4 storage compartment at the single molecule level, and our results suggest sortilin to be involved in the process of GLUT4 storage compartment formation during differentiation.

1871/B250
The Dynamic Nature of Vaults.
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Vaults are barrel-shaped nanocapsules with overall dimensions of $72.5 \times 41 \times 41$ nm, found in nearly all-eukaryotic cells. The interior volume is estimated at approximately $5 \times 10^7$ nm$^3$, large enough volume to encapsulate hundreds of proteins. The exterior shell is extremely thin (~20 nm), made of 78 - 96 copies of the major vault protein (MVP). The objective of this research is to investigate the dynamic nature of vaults both in Vitro and In Vivo in an effort to understand the kinetics of vault assembling/disassembling. In Vitro immuno-pulldown assays revealed that MVP subunits from mixed recombinant vaults appeared to be exchanged in the presence and absence of soluble cellular contents, suggesting this process was spontaneous and no additional accessory factors were required to exchange MVP subunits. After two adjacent cells, expressing N-terminal fused CFP and YFP vaults separately, were fused by PEG in approximately 2-3 hr, FRET (~30%) was observed from this newly formed hybrid cell, indicating vaults also exchanged their MVPs In vivo. Further investigation into the mechanism of this exchange demonstrated vaults were capable of separating themselves along the waist and rapidly reassembling back into whole hybrid vaults, supporting a half vault exchange mechanism. In Vivo mobility of vaults in mammalian cells was also examined by engineering vaults with a photoactivable GFP (PAGFP) to either the N- or the C- terminus of MVP. Single pixel photoactivation of PAGFP revealed rapid diffusion rates of vaults in the cytoplasm. FRAP experiments, designed to measure In Vivo mobility of expressed PAGFP vaults, showed no difference when the cells were pre-treated under various conditions. In conclusion, we explored the dynamic nature of vaults, suggesting a mechanism whereby vaults can functionally interact with the cellular milieu. (Supported by Mathers Foundation 4095186 and UC Discovery BIO-07-10671)

1872/B251

Biased Motor-assisted Flux of Soluble Cargoes Moving in Slow Axonal Transport.
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Slow axonal transport conveys soluble proteins along axons in a rate-class called Slow Component-b (SCb). Previous in-vivo radio-labeling studies have shown that soluble SCb cargoes are transported as discrete radioactive peaks, at rates 100-300 times slower than rates of fast vesicular transport. However, mechanisms by which soluble cargoes are coherently transported in axons are unclear. Using photoactivatable probes, we activated a discrete population of the SCb protein synapsin in axons of cultured hippocampal neurons, and studied its mobility over time with live-imaging. We found that synapsin population moved at rates similar to known in-vivo SCb transport velocities with kinetics that resembled diffusion, but had a distinct anterograde bias; very different from the known stochastic, one-at-a-time cargo dynamics of other rate-classes. Inhibition of motors greatly diminished the magnitude of synapsin movement, and eliminated the anterograde bias. Detailed biophysical modeling showed that overall synapsin movement has components that are both diffusive and motor-dependent, and inhibition of motors renders this movement largely diffusive. Rapid imaging of the leading anterograde edge of the photoactivated synapsin population revealed that individual synapsin particles coalesced and dispersed continuously, likely giving rise to the inefficient and sluggish movement of the overall synapsin population. We propose that soluble SCb cargoes are transported by a previously unknown mechanism in which molecular motors facilitate the diffusion of soluble cargoes by transiently transporting clusters of SCb cargo complexes; creating an anterogradely biased flux of the entire SCb population.

1873/B252

Simple Computer Model Reveals Posibility of Non-Motor Microtubule Binded Gradient Transport.
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Cellular microtubules are highly dynamic structures. In a way, plus ends (and GTP caps) are dynamically being distributed across the cytoplasm, creating some sort of GTP cap gradient. Specific type of intracellular transport that is based not on a motor proteins activity but on the binding of cargo molecules by microtubules plus ends' GTP cap has been hypothesized. This hypothesis is based on two assumptions: that significant gradient of plus ends exists in cytoplasm and that gradient of binding forces in its turn creates gradient of particles that in their normal state undergo Brownian motion. A simple computer model has been developed to test this theory. This model concludes a cytoplasm space - a two-dimensional circle area, stochastically moving particles, gradient of probabilistic binding forces, and straight microtubules, modeled by variety of probabilities of polymerization, depolimerization and GTP hydrolysis. The model has shown that both assumptions are, in fact, correct, and that unusual type of transport can be carried out by the system of dynamic microtubules and some binding forces provided by theirs GTP caps. It also helps to understand dependencies between different system parameters and properties of this transport type.

**1874/B253**  
**Diffusion in Cytoplasm: Effects of Excluded Volume Due to Internal Membranes and Cytoskeletal Structures.**  
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The intricate geometry of cytoskeletal networks and internal membranes causes the space available for diffusion in cytoplasm to be convoluted, thereby affecting macromolecule diffusivity. We present a first systematic computational study of this effect by approximating intracellular structures as mixtures of random overlapping obstacles of various shapes. Effective diffusion coefficients are computed using a fast homogenization technique. It is found that a simple two-parameter power law provides a remarkably accurate description of effective diffusion over the entire range of volume fractions and for any given composition of structures. This universality allows for fast computation of diffusion coefficients, once the obstacle shapes and volume fractions are specified. We demonstrate that the excluded volume effect alone can account for a four-to-sixfold reduction in diffusive transport in cells, relative to diffusion in vitro. The study lays the foundation for an accurate coarse-grain formulation that would account for cytoplasm heterogeneity on a micron scale and binding of tracers to intracellular structures.

**Cytoskeletal Organization II (1875 – 1894)**

**1875/B254**  
**Structure of the Ventral Disc of Giardia intestinalis.**  
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Giardia intestinalis is a ubiquitous unicellular, flagellated intestinal parasite of mammals with a two-stage lifecycle. Giardiasis is caused by ingestion of the cyst form, which then excysts in the duodenum into the trophozoite form that directly attaches to the intestinal epithelium via an organelle called ventral disc (VD), a complex spiral array of microtubules and associated structures. Giardia is a substantial health issue not only in the third world, but also in more developed countries including the U.S.A. We have analyzed the 3-D structure of the VD by cryo-electron tomography (cryo-ET) and subsequent volume-averaging procedures. These data revealed new insight into the building plan of the VD. An integral part of the VD are highly regular arrays of microtubules forming a spiral-like structure and connect to the lower cell membrane of the VD that, in an attached state makes contact with the intestinal microvilli. Each of these microtubules is connected to a sheet-like structure (microribbons) forming a parallel pattern of walls composed by regular building blocks forming a 16-nm repeat (exactly twice that of the αβ-tubulin repeat along a microtubule protofilament) along the microtubules. The current challenge
now is to dissect the large amounts of mainly unknown densities surrounding the microtubules, including the microribbons and laterally bridging elements. These bridging structures appear to regulate the lateral spacing between sheets that could lead to a VD deformation that modulates its attachment to the microvilli surface. Their variable extent suggests an arrangement by flexible coiled-coil elements, testable by sequence analysis of potential candidates. The identification of microtubule associated densities is currently in progress by several approaches: A, we will compare averaged 3-D data of wildtype VD’s with VD’s where a single component has been exchanged with a GFP-mutant. The GFP density will be large enough to be detected by statistical 3-D difference mapping between datasets with and without the GFP mutant. B, we check for kinesin like proteins and giardins in the close vicinity of the microtubules by immunolabeling with antibodies against GFP or kinesin head domains.

1876/B255
**Dynamin2 GTPase Regulates Focal Adhesions and the Actomyosin Cytoskeleton.**

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The large GTPase dynamin regulates endocytosis and the actin cytoskeleton. Although the mechanisms by which dynamin facilitates endocytic processes have been well studied, the mechanisms by which dynamin influences the cytoskeleton are not known. Our recent work supports the hypothesis that dynamin2 regulates the cytoskeleton via a GTPase-dependent actin filament remodeling activity. Dynamin2-dependent actin filament remodeling In Vitro required the actin binding- and dynamin2-binding protein, cortactin and conferred sensitivity of crosslinked actin filaments to severing by cofilin. Consistent with this hypothesis, depleting dynamin2 perturbed the global organization of actomyosin and of the actin-crosslinking protein, α-actinin, in U2OS cells (Mooren, et al. (2009) JBC, in press). In addition, pharmacological inhibition of dynamin2 using Dynasore reduced the association of the mechanosensitive protein, zyxin, with focal adhesions and actomyosin structures, suggesting that actomyosin-dependent contractility was decreased. To determine if regulation of actomyosin by dynamin2 also modulates the dynamic behavior of focal adhesions, we quantified the rates of assembly and disassembly of focal adhesions in HT1080 cells expressing GFP-paxillin and depleted of dynamin2. Depleting dynamin2 had no effect on the rates at which focal adhesions assembled or disassembled, but the lifetime of adhesions increased ~1.3-fold. Thus, we conclude that dynamin2 regulates focal adhesion turnover. Because focal adhesion turnover depends on Myosin II-dependent contractility and actomyosin organization is perturbed in dynamin2-depleted cells, we speculate that dynamin2 influences focal adhesion lifetimes via GTPase-dependent actin filament remodeling that promotes formation of contractile actomyosin arrays.

1877/B256
**Automated Estimation of the Spatial Distribution of Microtubules from 3D Confocal Microscopy Images.**

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Microtubules are polymeric structures that play important roles in many processes including cell division, intracellular transport, and cellular movements. Understanding the structure and function of microtubules in different cell types, under different conditions (i.e. presence of drugs) could enable many important discoveries in the health sciences. Currently, however, there is no established procedure for extracting basic physical information (such as the number of filaments, their lengths, etc.) from microtubule distributions of live cells in a high-throughput manner. We have developed a generative model-based approach that can quantify such spatial distribution parameters including number and mean of length distribution of microtubules directly from GFP-
tagged fluorescence confocal microscopy images. Our method is based on generating synthetic images of microtubule patterns according to a set of parameters and comparing these to real input images. We have performed experiments with real and simulated data to determine the accuracy of our model based estimation procedure. Results with simulated data show that the number and mean length of microtubules can be estimated with surprisingly high accuracy (mean absolute percentage error = 10% for number of microtubules and 22% for mean of length distribution of microtubules). Results with a set of 15 GFP-tagged intact HeLa cells show that the number of microtubules range from 50 - 175, and the mean lengths range from 25 - 75 microns, which is consistent with estimates (acquired using alternative means) described in the current literature. The work we describe will also have important applications in subcellular location proteomic studies. Future work includes combining our modeling procedure with models for other proteins. In addition, we also plan to extend the methodology to be able to estimate and model other filament-type proteins (e.g. actin) inside live cells.

1878/B257
Tubulin Post-Translational Modifications Mark Discrete Subpopulations of Microtubules in Epithelial Cells and May Indicate Functionally Distinct Microtubule Networks.
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In order to maintain discrete subcellular domains, such as those seen in neurons and polarized epithelial cells, the delivery of material needs to be precisely controlled within the cell. One hypothesis is that certain microtubule motors preferentially move along polymers that have been modified post-translationally. However, little is known about the distribution and molecular characteristics of these tracks in the cell. Here we have examined the microtubule network in the MDCK model of polarized epithelial cells. Before and after polarization, MDCK cells have well-organized microtubule arrays with subpopulations of acetylated microtubules, detyrosinated microtubules and poly-glutamylated microtubules. Although there is some overlap, these post-translational modifications (PTMs) mark distinct populations of microtubules. In polarized cells, this difference is even more striking as microtubules with different PTMs localize to distinct subcellular domains. The relative proportion of each PTM (to the total tubulin population) changes as cells become polarized. Microtubules marked by different PTMs are also differentially sensitive to nocodazole-induced and cold-induced depolymerization and to disruptions of Rho family GTPases. Together these data suggest that PTMs mark distinct populations of microtubules, which differ in their abundance, localization, stability and regulation by GTPases. These data also indicate that microtubule subpopulations change as cells become polarized, suggesting a mechanism by which molecular motors such as dynein and kinesins can target specific subcellular domains in spatially complex cells.

1879/B258
Op18 Reveals the Contribution of Non-Kinetochore Microtubules to the Dynamic Organization of the Vertebrate Meiotic Spindle.
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Accuracy in chromosome segregation depends on the assembly of a bipolar spindle. Unlike mitotic spindles, which have roughly equal amounts of kinetochore microtubules (kMTs) and non-kinetochore microtubules (non-kMTs), vertebrate meiotic spindles are predominantly comprised of non-kMTs, a large subset of which forms an anti-parallel 'barrel' array at the spindle equator. While kMTs are needed to drive chromosome segregation, the contributions of non-kMTs are more mysterious. Here, we show that increasing the concentration of Op18/stathmin, a component of the chromosome-mediated microtubule formation pathway that directly controls microtubule dynamics, can be used to deplete non-kMTs in the vertebrate meiotic spindle.
assembled in Xenopus egg extracts. Under these conditions, kMTs and the spindle pole associated non-kMT arrays persist in smaller spindles. In excess Op18, distances between sister kinetochores, an indicator of tension across centromeres, remain unchanged, even though kMTs flux poleward with a ~30% slower velocity and chromosomes oscillate more than in control metaphase spindles. Remarkably, kinesin-5, a conserved motor protein that can push microtubules apart and is required for the assembly and maintenance of bipolar meiotic spindles, is not needed to maintain spindle bipolarity in the presence of excess Op18. Our data suggest that non-kMTs in meiotic spindles contribute to normal kMT dynamics, stable chromosome positioning, and the establishment of proper spindle size. We propose that without non-kMTs, metaphase meiotic spindles are similar to mammalian mitotic spindles, which balance forces to maintain metaphase spindle organization in the absence of extensive anti-parallel microtubule overlap at the spindle equator or a key mitotic kinesin.

1880/B259
Small Molecule Regulators of the Rho-Controlled Diaphanous-related Formin Autoinhibitory Mechanism.
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Acting as effectors for Rho small GTP-binding proteins, the mammalian Diaphanous-related (mDia) family of formins nucleate and processively elongate actin and modulate microtubule stabilization to generate essential cytoskeletal structures underlying cell adhesion, migration, and division. Here, we present data implicating the formins as attractive targets for small-molecule intervention—not for inhibition, but for hyperactivation. The rationale for an activation versus inhibition strategy is four-fold. Rho proteins activate mDia proteins by direct binding and disruption of the interaction between the Dia-inhibitory (DID) and Dia-autoregulatory (DAD) domains flanking the actin-nucleating formin homology-2 (FH2) domain. In the past, we found that the autoinhibitory mechanism can be deregulated by DAD-derived peptides that bind to and disrupt DID-DAD interaction. The introduction of DAD peptides into breast and leukemic cancer cells triggers the stabilization of actin and microtubule dynamics, eventually leading to apoptosis. High-throughput screening for inhibitors of DID-DAD binding led to the identification of two distinct, but similar, semi-thiocarbazones called Intramimics (IMM)-1 and -2, for their ability to mimic intramolecular DAD binding. IMM In Vitro IC50 values (99 and 140 nM, respectively) were comparable to that of the DAD peptide (280 nM) itself. Both IMMs induced the expected stabilization of microtubule stabilization, induction of actin assembly, activated serum response factor (SRF)-regulated gene expression, and effectively triggered programmed cell death in a variety of tumor cell lines. IMMs should prove to be useful tool compounds in the study of formins in cell biology. Our goal is test IMMs in preclinical models for their ability to modulate myeloproliferation and to inhibit tumor growth, alone and in combination with other anti-tumor agents.

1881/B260
Centering of Dynamic Microtubule Asters by Cortical Pulling Forces.
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Dynamic microtubules (MTs) interact with the cortex to generate pushing and/or pulling forces that position organelles correctly with respect to the confining geometry of living cells. In particular, pulling forces mediated by cortex-attached dynein provide a versatile mechanism to properly position structures such as the mitotic spindle in systems ranging from small yeast cells to large embryonic cells. Nevertheless, the respective roles of pushing and pulling forces, and especially the mechanism by which pulling forces may contribute to centering processes, remain poorly understood. We address this question in an In Vitro experiment, where MT asters are
grown in microfabricated chambers. Pushing forces arise from MT polymerization and elastic restoring forces, and pulling forces are introduced by attaching dynein motor proteins to the chamber walls. Surprisingly, we find that MT asters center more reliably by a combination of pulling and pushing forces than by pushing forces alone. We explain our data with a theoretical model based on the following mechanism: slipping of growing MTs along the chamber walls generates an anisotropic distribution of MTs that, once MT ends are captured by dynein, leads to a reliable centering of the MT aster by pulling forces. We show that this mechanism is highly geometry-dependent, providing a possible explanation for the different positioning strategies employed in different cell types.

1882/B261
The γ-tubulin Complex Protein GCP4 Plays a Critical Role in Microtubule Organization in Arabidopsis thaliana.
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Microtubule (MT) nucleation and organization depend on the activity of the evolutionarily conserved protein γ-tubulin which forms a complex with five γ-tubulin complex proteins (GCPs). Functions of the non-core γ-tubulin complex components, namely GCP4-GCP6, are often essential for centrosome-based MT nucleation. However, it is unclear whether acentrosomal MT nucleation requires all GCP components in plant cells. In the model plant Arabidopsis thaliana, GCP4 was found to be associated with γ-tubulin in vivo. Because functional analysis of fundamentally important proteins like GCPs is often hindered by the lack of a viable mutant, an artificial microRNA construct (amiR-GCP4) was expressed in transgenic plants, aiming at repressing endogenous GCP4 expression. Phenotypes of drastic growth reduction were observed in these plants, and the degree of growth retardation was correspondent to the level of repression of GCP4 expression. It was also observed that sacrificing GCP4 depleted the γ-tubulin signal in the mitotic spindle and the phragmoplast. Consequently, MTs failed to converge at unified spindle poles, and the bipolar phragmoplast MT array frequently had discrete bundles with extended minus ends. Consequently, cell wall stubs were often observed in leaf epidermal cells reflecting failed cytokinesis. In addition, pavement cells of the leaf epidermal contained highly parallel MT bundles. Therefore, our results strongly support the notion that GCP4 is an indispensable component for the function of γ-tubulin in MT nucleation and organization, and ultimately for plant cell division and morphogenesis.

1883/B262
Coupling of the Dynamic Property of Septins with Their Enzymatic Activities.
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Septins are polymerizing GTP/GDP-binding proteins required for physical and functional organization of the cell cortex. As with actin and tubulin polymers, septin assembly is hypothesized to be governed by conformational changes coupled with nucleotide exchange/hydrolysis. However, this remains untested, partly because missense mutations introduced in septins’ G-domains have not given informative data so far. Here we show that two mammalian septin subunits that belong to the same structural subfamily behave differently in cells; i.e., they exhibit distinct bulk turnover rate when measured by FRAP (fluorescence recovery after photobleaching). The recombinant subunits exhibit distinct nucleotide preference and exchange kinetics. Analysis with chimeric subunits and swap mutants determine critical amino acid residues responsible for their distinct biochemical and dynamic properties. From these and other data, we propose that the enzymatic property of these subunits are critical determinants of the assembly/disassembly dynamics of septin filaments in cells and that similar septin subunits are not simply redundant but their unknown structural and biochemical differences contribute to the functional diversity of the septin system.
1884/B263
Podosome Dynamics in Vascular Smooth Muscle Cells Depends on CLASP-Associated Microtubules.
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Microtubules are essential regulators of invasive actin protrusions, podosomes, typical for matrix-degrading cells. The mechanism of this regulation is largely unclear. In this study, we addressed microtubule dependence of podosomes in vascular smooth muscle cells. Our results indicate that MTs strongly support podosome formation induced by protein kinase C (PKC) activation. On the contrary, when podosomes were induced by constitutively active oncogenic Src (caSrc), their existence and/or formation did not require MTs, suggesting that microtubules are involved in this pathway downstream of PKC but upstream of Src. Interestingly, caSrc-induced podosomes in the absence of microtubules were limited to restricted areas in a cell. Accordingly, in the absence of MTs active (phosphorylated) Src was particularly accumulated at these podosome formation sites, indicating that caSrc required microtubules for redistribution to new locations. Indeed, GFP-tagged c-Src was detected in microtubule-transported endosomes. We further identified that depletion of microtubule-associated proteins CLASPs mimicked effect of MT depolymerization on podosome dynamics and Src accumulation. We conclude that CLASPs are essential players in MT regulation of podosomes. Based on these data, we propose a role of CLASP-associated microtubules in distribution of Src Tyrosine Kinase to Regulate Podosome Dynamics in Vascular Smooth Muscle Cells.

1885/B264
Murine CENP-F Regulates Centrosomal Microtubule Nucleation and Interacts with Hook2 at the Centrosome.
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The microtubule network (MT) is essential in a broad spectrum of cellular functions. Many studies have linked CENP-F to MT-based activities as disruption of this protein leads to major changes in MT structure and function. Still the basis of CENP-F regulation of the MT network remains elusive. Here, our studies reveal a completely novel and critical localization and role for CENP-F at the centrosome, the major MT organizing center (MTOC) of the cell. Using a yeast two-hybrid screen, we identify Hook2, a linker protein that is essential for regulation of the MT network at the centrosome, as a binding partner of CENP-F. With recently developed immunochemical reagents, we confirm this interaction and reveal the novel localization of CENP-F at the centrosome. Importantly, in this first report of CENP-F/-/- cells, we demonstrate that ablation of CENP-F protein function eliminates MT repolymerization after standard nocodazole treatment. This inhibition of MT regrowth is centrosome-specific as MT repolymerization is readily observed from the Golgi in CENP-F/-/- cells. The centrosome-specific function of CENP-F in the regulation of MT growth is confirmed by expression of truncated CENP-F containing only the Hook2-binding domain. Further, analysis of partially reconstituted MTOC asters in cells that escape complete repolymerization inhibition, we show that disruption of CENP-F function impacts MT nucleation and potentially anchoring rather than promoting catastrophe. Our studies reveal a major new localization and function of CENP-F at the centrosome that is likely to impact a broad array of MT-based actions in the cell.

1886/B265
LOSK/SLK Kinase Is Essential for Intercellular Coordination and Directed Cell Locomotion in Experimental Wound.
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LOSK, Long Ste20-like Kinase (described also by other authors as SLK: Ste20-Like Kinase) is a member of the germinal center kinase group, identified as a microtubule and centrosome-associated protein. LOSK/SLK is ubiquitously expressed in mammalian cells, and its activity remains constant throughout the cell cycle though it slightly increases in mitosis. Either down- or up-regulation of this minor kinase results in apoptotic cell death, indicating the importance of cell functions regulated by LOSK/SLK. We have shown previously that LOSK/SLK activity inhibition by KR mutant expression or knock down results in disorganization of radial microtubule arrays, although both the microtubule-nucleating activity of the centrosome and Golgi apparatus integrity are not altered. Here we report that LOSK/SLK inhibition in cultured Vero cells (green monkey kidney) leads to the changes in cell/substrate and cell/cell interactions. It results in superposition of transfected cell lamella over the neighbors or the whole cell over the monolayer. The cells with inhibited LOSK/SLK exhibit reduced ability to move directly towards the experimental wound of the monolayer. The rate of lamella protrusion in such cells is not changed, however, cells are irregularly oriented, and it results in their retardation from the nearby moving cells. Notably, the focal adhesions of cells with inhibited LOSK/SLK revealed with immunostaining with anti-paxillin antibodies are not significantly changed. However, the dynamics of focal contacts estimated with GFP-paxillin expression is dramatically decreased. Therefore, LOSK/SLK might regulate the interaction of microtubules with focal adhesions, required for their disassembly.

1887/B266
Correlation of Cortical Microtubule Array Patterns with Cellular Morphogenesis in Arabidopsis thaliana Hypocotyl Cells.
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Cellular morphogenesis is central to the development of all multicellular organisms. Plant cells grow by producing a visco-plastic, extracellular matrix (the cell wall), and slowly extending that matrix using turgor pressure. Cortical interphase microtubule arrays direct cellulose microfibril deposition into the cell wall, providing a hypothesized mechanism for influencing cellular morphogenesis through creation of anisotropic wall material. To study the relationship between cortical microtubule array organization and cellular morphogenesis, we are analyzing microtubule array dynamics and cell expansion over extended time periods in living Arabidopsis cells using spinning disk confocal microscopy. Cortical microtubules typically form networks just beneath the cell surface that can organize into classes of patterns relative to the cell axis. Preliminary results suggest that the distribution and persistence of array patterns vary in hypocotyl cells relative to cell location and developmental stage. Array plasticity between patterns appears to fluctuate depending on developmental stage, suggesting active regulation related to cell wall organization. Microtubule nucleation site location and the initial trajectory of nascent microtubules appear to play a significant role in regulating overall array organization. We are characterizing local rates of cell surface expansion by observing the movement of fluorescent microspheres fixed to the cell wall. Quantifying growth patterns is revealing the developmental progression of local cell expansion rates within hypocotyl cells. Directly correlating cell wall growth with the underlying pattern of microtubules is providing crucial data for determining the degree to which microtubules control cellular morphogenesis.

1888/B267
Microtubule Dynamics and Function during HGF-induced Tubulogenesis in 3D Culture.
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Apical Basal polarized epithelial cells transition to a migratory phenotype during development and in tumor metastasis. This involves cell shape changes controlled by spatiotemporal regulation of cytoskeleton dynamics. Microtubules (MTs) are required for these changes in 2D, however, little is known about the role of MTs in more physiological 3D environments. To address this, we adapted the Madin Darby Canine Kidney (MDCK) epithelial morphogenesis model for high-resolution microscopy. MDCK cells embedded in extracellular matrix (ECM) form polarized...
Treatment of cysts with Hepatocyte Growth Factor (HGF) induces tubulogenesis, beginning with loss of cell polarity and migration into the ECM. We identified long basal extensions filled with dense MT bundles, indicating that MTs may be involved in this process. Plus-end tracking proteins (+TIPs) bind to plus-ends of growing MTs and regulate their dynamics and interactions with the cell cortex. The +TIP, EB1, acts as an adaptor for other +TIPs to recognize growing MT ends. Live cell imaging of MT dynamics using EB1-EGFP, revealed a large number of growing MTs in the extensions that displayed increased rates of growth and persistency in response to HGF. We hypothesized that MT stability and interactions with the tips of protrusions are required for the persistent formation of extensions. To test this, we used two approaches to disrupt +TIP complexes, through their dependence on EB1. First, we infected cells with a dominant-negative EB1 (EB1-C) adenovirus after cysts had formed. Following HGF stimulation, uninfected cysts displayed elongated extensions with lamella-like tips that pulled on and aligned the ECM. In contrast, EB1-C cysts displayed fewer, shorter basal extensions that did not remodel the matrix effectively. Second, we targeted EB1 for knockdown by shRNA. EB1 knockdown cells exhibited abnormal cyst morphology, with alterations in cell and lumen size and shape. We are currently characterizing these defects and determining the effects of EB1 knockdown on cytoskeleton dynamics in cysts responding to HGF. Together these data suggest that MTs play an important role in early HGF-induced tubulogenesis that is dependent on their regulation by +TIP complexes.

1889/B268
An RNAi Screen of Microtubule-Regulatory Proteins Identifies MARK2/Par1 as an Effector of Rac1-Mediated Microtubule Growth.
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Proper regulation of microtubule (MT) assembly dynamics is essential for directed cell migration. MT dynamics in migrating cells are spatially regulated by Rho GTPases. We have previously shown that activated Rac1 induces MT net growth by suppressing catastrophe and increasing growth velocity, and that Rac1 activity is required for polarized MT growth in the leading edge of migrating cells. We identified a necessary, but not sufficient, PAK kinase-mediated pathway downstream of Rac1 that promoted MT growth. Therefore, we hypothesized that additional factors promote MT net growth downstream of Rac1. To find these factors, we performed an RNAi screen in human U2OS osteosarcoma cells to determine if known MT-regulatory proteins were required for constitutively activated Rac1 promotion of MT growth. To analyze MT dynamics, we imaged fluorescent-tagged EB3, a MT plus-end binding protein that serves as a probe for the position of MT ends, and tracked the motion of EB3 comets in time-lapse movies using an automated computer program. Our results indicate that depletions of several MT-binding proteins change the growth rate of MTs in activated Rac1-expressing cells. We have focused on MARK2, a MT affinity-regulating kinase homologous to the C. elegans polarity protein Par1, whose depletion reduces the number of elongated MTs in the leading edge of Rac1-activated cells. We are currently testing how MARK2 is involved in promoting MT growth downstream of Rac1 and its requirement in cell migration.

1890/B269
Growth Rate Is Reduced and Optical Density Increased in Chlamydomonas Reinhardtii Microtubule-organization Mutant Strain cmu1-1.
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Wild-type Chlamydomonas reinhardtii cells are ovoid with paired anterior flagella. The mutant strain cmu1-1 has an altered cell shape with cells tending to be either tear-drop shaped or round. Additionally, this strain has a higher frequency of cells with aberrant numbers of nuclei and/or flagella. The change in cell shape correlates with a change in microtubule positioning within the cell body. Optical density was used to measure growth rates of cmu1-1 and wild-type cells. cmu1-1 growth rate is approximately 55% that of wild-type cells. This significantly decreased growth
rate could be due to cell lethality during interphase or could be due to defects in microtubule positioning during mitosis. In addition to changes in growth rate, \textit{cmu1-1} cells have an optical density approximately 140\% that of wild-type cells at the same cell density. Although in part attributable to multinucleate cells, the percentage of multinucleate cells in \textit{cmu1-1} cultures cannot completely account for this difference.

\textbf{1891/B270}

\textbf{Polarity Change in Fission Yeast: Modeling Possible Cytoskeletal Mechanisms for New End Take Off.}

\textit{T. Drake, D. Vavylonis; Physics, Lehigh University, Bethlehem, PA}

We develop theoretical models that describe the process of "new end take off" (NETO) in fission yeast. Fission yeast is a model organism for studying the cytoskeleton's role in cell polarization. Microtubules establish the cell tips, where they deliver cortical polarity factors such as Tea1p. These polarity factors mark sites for growth by recruiting formin For3p. In the beginning of the cell cycle, the cell grows at the old end, but then switches to bipolar growth during the G2 phase by initiating growth at the new end. During monopolar growth, microtubules grow symmetrically towards the two tips but actin and formin For3p are distributed asymmetrically. For3p locally nucleates actin cables. The cables aid transport of secretory vesicles and other cell polarity factors. Cables also contribute to localization of actin patches for endocytosis near cell tips. We investigated three models of NETO. for the first model, an autocatalytic mechanism reinforces actin polymerization at the cell tips. Saturation limits runaway monopolar growth. This model exhibits a transition from monopolar to bipolar growth as cell elongation adds available actin. Multiple stable growth patterns coexist near the transition region. for the second model, cortical landmarks amplify local excitations in the actin distribution. for the third model, we examine the possibility of multiple stable concentration gradients across the long axis of the cell: actin and For3p are interdependent leading to multiple possible cytoplasmic gradients with cell length-dependent stability. We compare to theoretical work by Csikasz-Nagy et al. [\textit{Yeast} 25:59 (2008)] and suggest experiments to distinguish among these mechanisms using actin depolymerizing drugs and genetic modifications.

\textbf{1892/B271}

\textbf{Model of Rho-Mediated Myosin Recruitment to the Cleavage Furrow during Cytokinesis.}

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The formation and constriction of the contractile ring during cytokinesis depends on the recruitment of myosin-II to the cell’s equatorial region. In recent experiments, Zhou and Wang [\textit{Mol. Biol. Cell} 19:318 (2008)] observed that during cytokinesis, mitotic animal cells assembled myosin into foci throughout the cell cortex. No significant long range transport of cortical myosin was found; instead, myosin progressively disassembled at the flanking regions of the cell and assembled in the center. Myosin foci underwent rounds of assembly and disassembly in a decaying oscillatory manner seen most clearly at the cell periphery during the process. This process of myosin recruitment was dependent on myosin motor activity and the Rho proteins. Microtubules establish a spatial pattern of differential Rho activity through signaling pathways that initiate in the central spindle and astral microtubules [Murthy & Wadsworth, \textit{J. Cell Sci.} 121:2350 (2008)]. We propose a reaction-diffusion model for the dynamics of myosin and Rho proteins during cytokinesis to interpret these experimental observations. In the model, an external input modulated in space by the mitotic spindle activates Rho. Active Rho promotes myosin assembly into minifilaments at the cortex. Mechanical stress by cortical myosin causes disassembly of myosin minifilaments and active Rho. We propose a switch-like relation between active Rho concentration and rate of cortical myosin assembly. The results of the model agree with the rate of recruitment of myosin to the cleavage furrow and the decaying amplitude of the concentration oscillations in the cell periphery. Spatial extent, period and decay rates of myosin oscillations are calculated. The model predicts various regimes of equatorial myosin recruitment for different values of the control parameters.
1893/B272
A Screen for Genes Involved In Nuclear Morphogenesis during Assembly of the Motile Apparatus in Marsilea vestita.
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The male gametes of Marsilea vestita are coiled, multiciliate cells. The coil consists of an elongated nucleus laminated against a large mitochondrion and a microtubule ribbon. Each cell has 140 cilia that protrude from dorsal side of the coil; the basal bodies are attached to the microtubule ribbon in a network of dense, granular material. We are interested in how the developing sperm cells are able to form a coil shape during gametogenesis. The sperm cells are formed in the male gametophyte, which arises from a meiotic product known as the microspore. When dry microspores are placed in water, they will go through a series of 9 rapid mitotic division cycles creating 7 sterile cells and 32 spermatids. Next, the spermatids assemble basal bodies in the absence of preexisting centrioles, and then begin cytoskeletal assembly, which is followed by cell elongation and coiling. The entire process reaches completion in 11 hours and is regulated post-transcriptionally. In a search for gene products involved in the formation of the coil, we picked candidates from a cDNA library where homologs are known to be involved in nuclear movement and anchoring. We performed RNAi knockdowns using dsRNA and looked at the formation of the coil with anti-alpha-tubulin antibody and DAPI staining to visualize the microtubule ribbon and the elongating nucleus, respectively. MvU1626 contains the SUN domain, MvU2148 is homologous to Nudc and MvU2178 is homologous with Nudc domain-containing protein 2. When these components are silenced, the nucleus is less compact, coiling is affected but the microtubule ribbon is present. The knockdowns can also affect cell division during the formation of the spermatogenous cells. In severe division phenocopies, the nucleus is misshapen and alpha tubulin staining is greatly reduced. These results indicate that nuclear movement proteins are important for nuclear morphogenesis in the developing spermatozoid from a fern. Supported by NSF grant MCB-0720486 and DBI-0842525 to SMW.

1894/B273
Role of Desmosomes in Microtubule Reorganization in the Epidermis.
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Cell-cell adhesion is necessary for cell attachment and organization of the cytoskeleton. One such cell-cell adhesion structure is the desmosome. It has traditionally been thought of as a static structure that anchors intermediate filaments. However, novel roles for desmosomes have emerged, including organization of the microtubule cytoskeleton and the actin cytoskeleton. One way in which desmosomes are thought to organize microtubules is through the recruitment of centrosomal proteins, such as ninein. Here, we show that a complex of proteins implicated in microtubule organization is recruited to the desmosome. These include the centrosomal proteins Lis1, Nudel, and the plus-end microtubule-binding protein CLIP170. In the epidermis, desmoplakin is required to recruit these proteins to the cell cortex, where they colocalize with desmoplakin and microtubules. We have found that only one of the naturally-occurring isoforms of desmoplakin, desmoplakin I, is able to recruit members of the complex. Furthermore, we report that the loss of one member of this complex, Lis1, results in fragile, thin skin, smaller desmosomes, and disorganization of the microtubule cytoskeleton. These changes result in a barrier defect, causing perinatal death. Our data suggests that desmoplakin is essential to recruit centrosomal proteins and microtubules to the cell cortex in the epidermis, and this is necessary to maintain proper epidermal structure and function.
Nerve Cell Cytoskeleton (1895 – 1914)

1895/B274
Disruption of Retrograde Axonal Transport of Endosomes in a P150/Glued Model of Motor Neuron Disease.
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Increasing evidence suggests that defects in axonal transport may be a primary cause of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS). In humans, a G59S mutation in the CAP-Gly microtubule-binding domain of the P150/Glued protein, a subunit of dynein, causes an autosomal dominant lower motor neuron disease with ALS-like pathology. Dynactin regulates dynein-mediated microtubule transport, however the function of the CAP-Gly domain in axonal transport is unclear. We have studied the effect of this G59S mutation in Drosophila using transgenic flies that overexpress wild type and mutant forms of Glued, and also a “knock-in” G59S allele (G38S in flies). Our genetic analysis suggests G38S causes a partial loss-of-function allele of Glued, but can also function in a dominant-negative fashion when overexpressed. G38S flies exhibit severe and progressive loss of locomotor function and early lethality, similar to phenotypes observed following overexpression of G38S mutant or dominant-negative Glued protein. Homozygous and hemizygous G38S animals have large axonal swellings that contain numerous membrane-bound vesicles. These vesicular structures contain synaptotagmin and are labeled with late endosomal and lysosomal markers. Live imaging of mitochondrial transport in G38S larval nerves suggests that processivity of retrograde axonal transport is disrupted, leading to an increase in the frequency of pauses and reversals and a decrease in “run” length and duration. Interestingly, the dynein retrograde motor accumulates on endosomal vesicles at terminal boutons of the neuromuscular junction (NMJ) in mutant animals, suggesting that the CAP-Gly microtubule-binding domain is important for the initiation of dynein-mediated retrograde movement at (+) ends of microtubules. Electrophysiological analysis at the larval NMJ reveals a presynaptic defect in neurotransmitter release, suggesting that defective endosomal trafficking at the nerve terminal leads to an impairment in synaptic vesicle exocytosis. Identification of suppressors of temperature-dependent paralytic phenotypes suggests that defects in endosomal sorting may be central to the pathogenesis of G59S-mediated motor neuron disease.

1896/B275
Role of Kinesin-5 in Neuronal Migration.
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Kinesin-5 (also called Eg5 or kif11) is a homotetrameric motor protein that interacts with adjacent microtubules, and is best known for its role in mitosis. In previous studies on post-migratory neurons, we found that depletion of kinesin-5 results in longer axons with an enhanced frequency in the bidirectional transport of short microtubules. Here we show that depletion or pharmacological inhibition of kinesin-5 results in migratory neurons that move faster and bear shorter leading processes. In addition, the directionality of migration is more random, and the distance between the centrosome and the nucleus is shorter. Live-cell imaging revealed the presence of short microtubules moving within the leading process. However, the frequency of these moving microtubules was less than in the axon, and the movement occurred only in the anterograde direction. Even so, the frequency of the transport was enhanced by inhibition of kinesin-5. 3D electron tomography was then used to provide more information on microtubule length as well as the distribution of microtubule ends in migratory neurons. Unlike the case in the axon, wherein microtubule are all free at both ends and exist in a variety of different lengths, most microtubules in the leading process extend its full length and remain attached to the centrosome. The phenotype observed with kinesin-5 inhibition suggests a significant role for this motor in
regulating neuronal migration. The unique features of the microtubule array of a migratory neuron compared to the axon provide insight on the mechanism by which kinesin-5 functions in each case.

1897/B276
Self-Organization of the *Escherichia coli* Chemotaxis Network Imaged with Super-Resolution Light Microscopy.  
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Eukaryotic cells contain spatially organized structures, such as membrane-bound organelles, which are necessary for cellular function. Prokaryotic cells lack similar higher-ordered structures but are still capable of organizing their interiors and segregating large protein networks. The chemotaxis network of *Escherichia coli* is one such highly spatially organized system of proteins, and is responsible for signal transduction and processing. Chemotaxis receptors assemble into large clusters containing tens of thousands of proteins which have been observed at cell poles and future division sites. Despite extensive study, it remains unclear how chemotaxis clusters form, what controls cluster size and density, and how the cellular location of clusters is robustly maintained in growing and dividing cells. Here, we use a super-resolution optical technique called photoactivated localization microscopy (PALM) to map the cellular locations of three proteins central to bacterial chemotaxis (the Tar receptor, CheY, and CheW) with a precision of 15 nm. We find that cluster sizes are approximately exponentially distributed, with no characteristic cluster size. One-third of receptors are part of smaller lateral clusters that have not been previously observed. Analysis of the relative cellular locations of 1.1 million individual proteins (from 326 cells) suggests that clusters form via stochastic self-assembly. The super-resolution PALM maps of *E. coli* receptors support a growing collection of evidence that stochastic self-assembly can create and maintain periodic structures in biological membranes, without direct cytoskeletal involvement or active transport.

1898/B277
Mixed Microtubules Steer Dynein-Driven Cargo Transport into Dendrites.  
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To establish and maintain their polarized morphology, neurons employ active transport driven by molecular motors to sort cargo between axons and dendrites. However, the basic traffic rules governing polarized transport on neuronal microtubule arrays are unclear. Here we show that the microtubule minus-end directed motor dynein is required for the polarized targeting of dendrite-specific cargo. To directly examine how dynein motors contribute to polarized dendritic transport, we established a trafficking assay in hippocampal neurons to selectively probe specific motor protein activity. This revealed that, unlike kinesins, dynein motors drive cargo selectively into dendrites, governed by their mixed microtubule array. Quantitative modeling demonstrated that bidirectional dynein-driven transport on mixed microtubules provides an efficient mechanism to establish a stable density of continuously renewing vesicles in dendrites. We propose that dynein establishes the initial sorting of dendritic cargo while additional motor proteins assist in subsequent delivery.

1899/B278
Inhibition of Kinesin-5 Enhances Axonal Regeneration over Inhibitory Proteoglycans Associated with Nerve Injury.  
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Following peripheral injury, neurons of the dorsal root ganglion have the capacity to regenerate axons. However, these axons fail to grow over repulsive molecules, such as chondroitin sulfate proteoglycans (CSPGs), secreted by the glial scar after injury. Kinesin-5 (also called kif11 or Eg5) is a homotetrameric motor protein that is highly expressed in developing neurons and serves as a “brake” on the microtubule array. During development, kinesin-5 has a role in reducing the rate of axonal growth and enhancing the frequency of axonal retraction. When kinesin-5 is inhibited, developing axons grow faster, retract less, and do not turn. Here we demonstrate that kinesin-5 is also highly expressed in adult neurons of both the central and peripheral nervous systems. We hypothesized that pharmacological inhibition of kinesin-5, using the compound monastrol, might enable injured adult axons to regenerate better and grow over repulsive molecules. Using cultures of adult mouse dorsal root ganglion neurons, we found that monastrol increased the frequency of axons growing onto CSPGs at a CSPG concentration of 50 \( \mu \text{g/ml} \). Monastrol is not effective at greater concentrations of CSPGs, but when combined with chondroitinase ABC to partially digest the CSPGs, monastrol results in more robust regeneration than observed with the chondroitinase ABC alone. These results indicate that pharmacological inhibition of kinesin-5 may be a useful tool to enhance axonal regeneration after injury, especially when used in combination with other available tools.

1900/B279
Kinesin-2 and the +TIPS EB1 and APC Direct Microtubule Growth to Generate Uniform Microtubule Polarity in Dendrites.
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In many differentiated cells microtubules are organized into polarized noncentrosomal arrays, yet few mechanisms that control these arrays have been identified. We have identified a novel mechanism that allows uniform microtubule polarity to be maintained in branched dendrites. Drosophila neurons have a highly polarized microtubule cytoskeleton in which both the axonal and dendritic microtubules are uniformly oriented. To maintain uniform microtubule polarity in dendrites, we hypothesized that the direction of microtubule growth must be controlled at branch points. All plus ends in these dendrites grow towards the cell body. If they grow through branch points, and growth is not directed, then uniform polarity would be disrupted as growing microtubules could turn either towards or away from the cell body at the branch. We use Drosophila dendritic arborization (da) neurons as our model system to test this hypothesis. Live, In Vivo imaging of EB1-GFP dynamics together with stable microtubules showed that growing microtubules navigate dendrite branch points by turning the same direction 98\% of the time, making use of stable microtubules as tracks. We find that kinesin-2 (KIF3), and the +TIPS EB1 and APC (adenomatous polyposis coli), are required for uniform microtubule polarity; when their levels are reduced by RNAi and/or in mutant animals, uniform microtubule polarity is lost specifically in dendrites. Velocity of plus end growth does not change, nor is microtubule polarity in axons altered when levels of kinesin-2 are reduced. Furthermore, we have found APC2-GFP to be distinctly localized to dendritic branch points. As kinesin-2 and APC are known to physically interact in other systems, we propose APC links kinesin-2 to tips of growing microtubules, and that the motor protein steers microtubule growth along existing microtubules at branch points. This is the only mechanism so far demonstrated to contribute to dendrite microtubule polarity in Drosophila, and guiding microtubule growth represents a novel role for +TIPs and kinesin-2.

1901/B280
Kinesin-5 Functions in the Transition Zone of Growth Cones to Regulate Microtubule Invasion into Filopodia.
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The growth cone, the motile tip of the axon, can be divided into a microtubule-rich central (C-) domain, an actin-rich peripheral (P) domain, and the transition (T-) zone between them. Selective invasion of microtubules from the C-domain into one side of the P-domain is critical for the growth
cone to turn in that direction. Previously, we showed that kinesin-5, a mitotic homotetrameric motor protein best known for its role in mitosis, is involved in the polarized distribution of microtubules in the P-domain and is thereby essential for growth cone turning. During mitosis, the association of kinesin-5 with microtubules is regulated by phosphorylation of kinesin-5 at T926 by CDC2. Based on immunocytochemical analysis of growth cones stained for total and phospho-kinesin-5, we proposed a model where kinesin-5 functions in the T-zone to inhibit microtubule invasion into the P-domain on the side opposite to the turn. Here, we tested this model using various experimental approaches. In a first set of studies, we demonstrate that CDK5, the neuronal homologue of CDC2, is the relevant kinase in neurons for phosphorylating kinesin-5 at T926, and that laminin, an extracellular cue relevant to growth cone turning, induces phosphorylation of kinesin-5 at T926 in a dose-dependent manner. The hypothesis that the polarization of kinesin-5 in growth cones is normally regulated by phosphorylation of kinesin-5 at T926 was supported by the results of studies using phospho-mutants of kinesin-5. Finally, we used a laser-based technology to focally inactivate kinesin-5 in the different regions of the growth cone. In support of our model, focal inactivation of kinesin-5 specifically in the T-zone increased the invasion of microtubules into the P-domain.

1902/B281
Disruption of Neurofilament Transport by a Hereditary Spastic Paraplegia Mutation in Kinesin-1A.
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Hereditary spastic paraplegia (HSP) is characterized by progressive degeneration of the corticospinal tracts, leading to lower limb spasticity and weakness. One of the dominantly inherited forms of HSP (SPG10) is caused by point mutations in kinesin-1A (formerly KIF5A), which is thought to be an anterograde motor for neurofilaments. This suggests that neurofilament transport may be disrupted in SPG10. To explore this hypothesis, we investigated the effect of an SPG10 point mutant, N256S-kinesin-1A, on neurofilament transport. Previous studies by the Woehlke lab have shown that this mutant has impaired velocity and processivity in vitro. We co-transfected cultured mouse cortical neurons by electroporation with GFP-tagged neurofilament protein M (GFP-NFM), with or without mutant or wild type kinesin-1A, and then analyzed neurofilament transport in axons using live-cell time-lapse imaging after 8-12 days in culture. The N256S mutant decreased anterograde neurofilament transport flux (from 136 to 35 µm/axon/hr) by decreasing anterograde transport frequency (from 3.9 to 1.0 moving filaments/hr) without any statistically significant effect on anterograde transport velocity. Consistent with our previous observations on neurofilament transport in kinesin-1A knockout neurons, retrograde neurofilament transport flux was also decreased (from 111 to 85 µm/axon/hr). This was due to decreased retrograde transport frequency (from 3.1 to 1.9 moving filaments/hr) in spite of a statistically significant increase in retrograde transport velocity (from 0.32 to 0.41 µm/s). Wild type kinesin-1A reduced both anterograde and retrograde neurofilament transport flux to 83 and 95 µm/axon/hr, respectively, suggesting a partial inhibition of neurofilament transport, but this was not statistically significant. Thus overexpression of the wild type motor had some effect on neurofilament transport, but only the effect of the N256S mutant was statistically significant. These data suggest that neurofilament transport may be disrupted in patients with SPG10, and that disruptions in neurofilament transport may contribute to the etiology of this disease.

1903/B282
Kinesin-12 Interacts with Both Microtubules and Actin Filaments to Influence Axonal Growth and Navigation.
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We have determined that developing neurons express kinesins that are known to generate forces on microtubules during mitosis. Such forces are important for the microtubule arrays in the neuron to coordinate properly with actin-based compressive forces within the axonal shaft as well as the retrograde flow of actin filaments in the growth cone. Kinesin-12 (also called kif15) is particularly interesting in this regard, because data on its distribution in fibroblasts suggests that it may interact directly with actin filaments as well as microtubules. Kinesin-12 co-localizes with actin filaments during interphase and telophase, but with microtubules during the other phases of mitosis. In neurons, kinesin-12 co-localizes only with microtubules, but would presumably retain the capacity to interact with actin filaments in functionally important ways. Here, we have documented, using biochemical approaches, that kinesin-12 indeed has the ability to interact directly with actin filaments. In primary cultures of rat sympathetic neurons, knockdown of kinesin-12 results in faster rates of axonal growth and inhibition of growth cone turning. Live-cell imaging reveals abnormalities in the tracking behavior of microtubules along actin filaments in the filopodia of the growth cone. In the axons of cultured cortical neurons, depletion of kinesin-12 results in a severe diminution in the number of “growth cone like waves” within the axon, which is a phenomenon involving the movement of actin filament assemblies relative to microtubules. We propose that kinesin-12 plays important roles in regulating axonal growth and navigation by functionally linking microtubules and actin filaments via force generation.

1904/B283
Centrosomes are not Required For the Establishment and Maintenance of Neuronal Microtubule Organization.
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In many proliferating and undifferentiated animal cells, microtubules are nucleated by centrosomes. These organelles organize microtubules into radial arrays, with minus ends that remain anchored. However, in certain differentiated cells, such as neurons and epithelial cells, noncentrosomal arrays of microtubules are found. These arrays have been implicated in the specific functions of these differentiated cells, such as protein transport and cell morphogenesis. Drosophila, which has a highly polarized arrangement of microtubules in neurons, provides a good model for the study of microtubule polarity and the organization of noncentrosomal arrays. Microtubules of Drosophila dendrites are primarily oriented with their minus ends distal to the cell body, while microtubules in axons are primarily plus-end out (Stone et al 2008, Mol Biol Cell 19: 4122-4129). The objective of this study is to determine whether the centrosome is essential in the establishment and maintenance of microtubule arrays. To examine the role of the centrosome, immunostaining experiments were performed, and microtubule dynamics were analyzed using the plus-end tracking protein EB1. Immunostaining results show that gamma-tubulin, a component of the centrosome, is not localized at the centriole during axon outgrowth in embryonic motor neurons. Additionally, the centriolar protein Fzr varied in location, and did not correspond to the site of axon outgrowth. In the imaginal wing discs of prepupae, axon outgrowth of sas4 mutants, which lack centrioles, also occurs normally. EB1-GFP dynamics were examined in Drosophila embryos; EB1-GFP comets were found to originate at sites not associated with the centriole, indicating that microtubules can be nucleated away from the centriole. In larval neurons, microtubule dynamics and orientation were similar in sas4 mutants and wild type animals. Based on these results, we conclude that the centrosome is not essential for neuronal microtubule organization.

1905/B284
Actin Isoforms in Neuronal Development and Function.
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The actin cytoskeleton plays a well characterized role in neuronal development and function, but less is known about how the specific isoform composition of actin affects its function. Mammalian neurons express two actin isoforms, $\beta_{cyto}$- and $\gamma_{cyto}$-actin, which differ by only four out of 375
amino acids. Despite their near identical amino acid composition, numerous In Vitro studies have suggested that the two isoforms have unique functions in neurons based on distinct subcellular localizations. β_cyt-actin is enriched at the leading edge of growth cones via an mRNA local translation mechanism that is essential for growth cone guidance and axon elongation, while γ_cyt-actin is not known to be targeted or directly involved in axon guidance. Indeed, primary hippocampal neurons cultured from Actg1-null embryos differentiate normally and adult Actg1-null mice exhibit no obvious CNS defects. To assess the importance of β_cyt-actin in neurons in vivo, we have used transgenic Cre lines to generate motor-neuron specific (MNs-ActbKO) and central nervous system (CNS-ActbKO) β_cyt-actin knock-out mice. Surprisingly, MNs-ActbKO mice develop normally and perform similarly to control littermates in muscle function tests at six months of age with no sign of muscle denervation or neuromuscular junction defects. CNS-ActbKO mice are born at the expected rate but are smaller in size and exhibit maternal behavior defects such that no pups born to a KO mother live longer than one day, a phenotype not observed in Actg1-null mothers. Thus, we conclude that the functional importance of β_cyt-actin may be more restricted to select cell types in the CNS than previously thought.

1906/B285
Protein Kinase C Effects on Actin Filament Dynamics and Branching in Neuronal Growth Cones.
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Protein kinase C (PKC) is known to alter cell structure, however, underlying effects on cytoskeletal protein dynamics are not well understood. We have investigated potential mechanisms underlying PKC dependent changes in the actin cytoskeleton in neuronal growth cones. Phorbol 12,13-dibuterate (PDBu) was used to activate PKC in Aplysia bag cell neurons followed by quantitative analysis of actin filament dynamics using fluorescent speckle microscopy (FSM) and computer-assisted particle analysis before and after drug treatment. Corresponding structural effects were assessed at light microscopic and ultrastructural levels. PKC activation resulted in expansion of the peripheral (P) domain accompanied by elongation and merging of radial filopodial actin bundles, whereas the central organelle-rich cytoplasmic (C) domain tended to decrease in area. Interestingly, net actin content in the P domain was markedly decreased (to 48% of initial values) accompanied by an apparent elimination of short branched networks (assessed by ultrastructural analysis). Peripheral retrograde actin filament flow rates were unchanged. PKC activation appeared to bias formation of long actin bundles in the P domain. Preliminary analysis suggested a decrease in actin assembly and free barbed end density near the leading edge. Our results suggest that PKC activation selectively depletes branched short filament networks from the growth cone periphery. This phenotype may result from a change in the number and localization of free barbed ends possibly via signaling to regulators of actin nucleation and recycling.

1907/B286
The PI3K-AKT Pathway and the Arp2/3 Complex are Required for NGF-Induced Axonal Filopodia Formation.
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Neuronal axons generate axon collateral branches that are required for the formation of correct patterns of innervation. External cues from the surrounding environment are responsible for establishing these neuronal connections. The first step in the formation of axon collateral branches involves the extension of a filopodium from the axon. Nerve Growth Factor (NGF) induces filopodia and collateral formation along the axon shaft of sensory neurons. Axonal filopodia are formed from precursor F-actin based structures termed “patches”. The mechanisms regulating the formation of NGF-induced axonal filopodia are poorly understood. We report that NGF increases the frequency of axonal filopodial formation by increasing the formation of patches
without altering their lifespans or the probability that a patch will give rise to a filopodium. Activation of PI3K with a cell permeable peptide increases patch formation while inhibition of PI3K using LY294002 blocks NGF-induced patch formation and filopodia formation along the axon shaft. Treatment with Akt Inhibitor VII inhibited NGF-induced patch formation and filopodia formation along the axon shaft. These data indicate that NGF-induced filopodia formation is a result of an increase in the frequency of F-actin patch formation mediated by the PI3K-Akt pathway. Furthermore, the p21-GFP subunit of Arp2/3, the F-actin nucleating system, is recruited to emerging patches. NGF increases levels of cortactin, an activator of Arp2/3, which targets to axonal patches. Inhibition of Arp2/3 function using the CA-domain of N-WASP decreased the numbers and rates of spontaneous patch formation and axonal filopodia, and blocked NGF-induced patches and filopodia. These data demonstrate that Arp2/3 is required for the formation of axonal patches and filopodia under normal conditions and in response to NGF.

**1908/B287**

**Local Dynamics of Actin Polymerization Resolved within Dendritic Spines by Single-Molecule Tracking PALM.**

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Continuous actin polymerization within spines enables rapid morphological change that accompanies many forms of synaptic plasticity and behavioral learning. Beyond control of spine morphology per se, actin serves multiple roles in spines to regulate synapse function. However, because of the submicron dimensions of spines, the arrangement of dynamic actin filaments within them is unknown, and most notably it is not clear whether regulation of actin organization at the synapse is unique or common to the spine as a whole. To map sites of actin polymerization within single spines, we first developed a high-resolution, optical monomer incorporation assay. This revealed that in the spines of cultured hippocampal neurons, a net inward actin flow oriented toward the base of the spine is driven by spatially heterogeneous ongoing polymerization rates at the spine head membrane, with faster fractional replacement of existing polymerized actin molecules occurring at the spine tip. By expressing photoswitchable actin along with markers of the PSD, we measured the movement of individual actin molecules within living spines using single-particle tracking photoactivated localization microscopy (sptPALM). Rapid, polymerization-driven actin flow along single filaments in spines often exceeded flow velocity measured at the leading edge of migrating cells, suggesting a highly active actin network in the spine. By assembling numerous single-molecule tracks, we mapped the spatial organization of filament dynamics within single spines and with respect to the synapse. This approach revealed inward actin flow from broad areas of the spine plasma membrane, a dense central core of heterogeneous filament orientation, and actin filaments in the spine neck oriented away from the spine head. Surprisingly, the velocity of single actin molecules along filaments was elevated both near the postsynaptic density and at discrete points along the spine membrane distant from the synapse. We conclude that actin polymerization is initiated and independently regulated at many well-separated foci within spines. This organization may be necessary for the specific adjustment of synaptic molecular content that underlies functional plasticity.

**1909/B288**

**Distinct Roles of C-Jun NH2-Terminal Kinase (JNK) Isoforms in Initiation and Elongation of Regenerating Axons: Effects on Cytoskeleton Reorganization.**

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C-jun NH2-terminal kinases (JNKs) are members of the MAP kinases family, comprising three isoforms (JNK1, JNK2 and JNK3) activated in response to various stimuli including growth factors
and inflammatory cytokines. During nervous system development, JNKs are involved in neurite growth and might also play a function in neuronal network formation while in the adult, a role in maintaining neuronal cytoarchitecture has been suggested. After injury, adult peripheral neurons initiate complex signaling pathways that converge to regulate cytoskeleton dynamics which is essential for neuronal regeneration. In regenerating adult dorsal root ganglions (DRG) neurons, it has been shown that JNKs inhibition results in a defect on neurite initiation. Here, we further investigated the role of JNKs activation and the specific function of each isoform during adult axonal regeneration and plasticity. Particularly, we examined putative molecular effectors of JNKs, such as the microtubule-associated protein 1B (MAP1B) whose function is regulated by phosphorylation (MAP1B-P) and for which we have previously demonstrated a role in axonal regeneration (Bouquet et al., 2004, J Neurosci.; MCN, 2007). Pharmacological (using a specific JNK inhibitor) and genetic (using JNK “knock-out” mice) approaches show sustained JNK activation in regenerating DRG neurons and reveals that signaling by individual JNKs is differentially implicated in regulating cytoskeleton reorganization.

1910/B289
14-3-3 Proteins Regulate Axonal Growth Cone Responses by Regulating PKA Activity.
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The growth cone is a critical structure regulating the speed and direction of neuronal outgrowth during development. How the growth cone spatially and temporally regulates signals from guidance cues is not fully known. Through a proteomic analysis of a mechanically purified growth cone preparation from E6 chick RGCs we identified several isoforms of the 14-3-3 family of adaptor proteins as major constituents of the growth cone. 14-3-3 proteins bind and regulate the activity of multiple proteins through interactions with phospho-serine and phospho-threonine containing motifs. By immunofluorescence we show that 14-3-3 isoforms are expressed in growth cones of many types of neurons and that their expression is developmentally regulated. Using the 14-3-3 antagonist R18 or miRNA-mediated knockdown of individual 14-3-3 isoforms we find that 14-3-3s switch nerve growth factor-dependent repulsion to attraction in E13 chick and P5 rat DRG neurons. This switching effect is reminiscent of switching responses observed in response to regulating levels of cyclic nucleotides. Intriguingly, R18-dependent switching is blocked by inhibitors of PKA but not by the cAMP antagonist Rp-cAMPS indicating that 14-3-3 proteins may directly regulate PKA. Consistent with this model we find that specific 14-3-3 isoforms interact with the PKA regulatory subunit. Further, R18 transfection into PC12 cells results in a dissociation of the regulatory and catalytic subunit of PKA suggesting that 14-3-3 heterodimers stabilize the PKA holoenzyme. Together our data indicates that 14-3-3 proteins play a critical role in modulating growth cone response to extracellular cues through regulating PKA and associated downstream signaling.

1911/B290
New Roles for an Old Giant: Novel Interactions of the Dystonin Protein.
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Dystonin Bpag1 is a large cytoskeletal plakin protein that contributes to subcellular structure. Loss of dystonin in mice results in neuromuscular dysfunction and early death in a mouse mutant called dystonia musculorum. The phenotype in these mice is caused by a sensory neuropathy that results in abnormal posturing, uncoordinated muscle movements and poor muscle tone. The multi-domain structure of dystonin isoforms allows for their participation in numerous cell-type specific interactions. Previous characterization of the non-epithelial dystonin proteins has focussed on the alternately spliced N-terminal actin-binding domain and the C-
terminal microtubule-binding domain. Interactions of the family-defining plakin domain of these proteins, however, have not been explored in detail. The purpose of the present study was to identify novel interacting partners of the plakin domain of the neuronal isoform of dystonin (dystonin-a). To this end, pull-down interaction analyses were performed and several candidate interacting partners were identified. The legitimacy of the candidates was validated through co-immunoprecipitation, co-immunofluorescence and proximity ligation assays. MAP1B was one such candidate that demonstrated a strong interaction with neuronal dystonin. CRMP2 and Myosin IIb were also identified as potential binding partners of the plakin domain of dystonin-a. These identified partner proteins show associations with components of the cytoskeleton, further supporting dystonin's role as a cytoskeletal linker protein. Additionally, the identification and validation of a strong interaction with clathrin heavy chain, the major component of the clathrin triskelion involved in intracellular transport and endocytosis, has raised possibilities for new, previously undiscovered roles for dystonin. Supported by funds from the Canadian Institutes of Health Research.

**1912/B291**

**Calmin Is required for atRA-mediated Neurite Outgrowth In Murine Neuro2a Cells.**

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The vitamin a metabolite, all-trans retinoic acid (atRA), influences the growth and differentiation of many cell types, including those in the developing nervous system (reviewed in Clagett-Dame et al., 2006, J. Neurobiol. 66:756). atRA acts by binding to the retinoic acid family of nuclear receptors (RAR) to regulate transcriptional activity within the cell. Using a subtracted cDNA library constructed from the human SH-SY5Y neuroblastoma cell line, several atRA-responsive genes were identified, including **calmin (Clmn)**, also known as retinoic acid induced in neuroblastoma 12, or RAINB12 (Merrill et al., 2004, Biol. Chem. 385:605). Our group initially observed that **Clmn** expression in early embryos is sensitive to retinoid status. In vitamin A-deficient embryos, **Clmn** mRNA is dramatically downregulated in the developing neuroepithelium adjacent to the paraxial mesoderm, whereas expression is unchanged in the developing head and tail regions. **Clmn** is expressed in murine Neuro2a (N2A) cells that are also induced to differentiate in response to atRA. N2A cells treated with atRA for 48h show an increase in neurite outgrowth that is eliminated when **Clmn** is knocked down in RNA interference studies. Additionally, when exogenous CLMN is reintroduced into N2A cells 24h prior to atRA treatment, there is a partial rescue of neuronal elongation, supporting the observation that **Clmn** knockdown is responsible for the loss of neurite outgrowth. Future studies will be directed at assessing whether CLMN acts by interacting with the actin cytoskeleton and/or microtubule network or whether it could work at an earlier stage, coordinating cell cycle exit and neuronal differentiation. Based on our studies, we conclude that the atRA-responsive gene **Clmn** is required for neurite outgrowth in cultured neuroblastoma cells.

**1913/B292**

**C. elegans Septins Function in Axonal Outgrowth and Stability.**

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Septins, a family of cytoskeletal filament-forming GTPases, are linked to several neuropathologies, however their roles in nervous system development and function are not understood. The simplicity of the worm septin complement, with only two septin genes, suggests that **C. elegans** might be particularly valuable for dissection of conserved In Vivo roles for animal septins in the nervous system. We previously found that the two worm septins, UNC-59 and UNC-61, play critical roles in nervous system development. Septin mutations cause locomotory behavior defects in newly hatched larvae in the absence of cytokinesis failures. The likely cause of the uncoordination is variable axonal migration defects in ventral cord motor neurons in the
mutant larvae. Blebbed neurons, resembling degenerating neurons observed in other systems were also observed in septin mutant larvae. These results suggest that septins are important for axon outgrowth and stability. In this study, C. elegans primary embryonic neuronal cell cultures were used to analyze neuronal processes under conditions in which septin function was impaired by genetic mutations, RNAi, or treatment with the septin drug forchlorfenuron. Septin mutations or treatment of neurons with septin dsRNA resulted in reduced numbers of cells with axons and shorter average axon lengths. Although fewer septin dsRNA-treated cells extended neurites, those that did extend processes had increased numbers of neurites per cell, suggesting compromised cell polarity. Treatment with forchlorfenuron resulted in rapid, but reversible, axon loss. These data support roles for septins in initiation and/or promotion of neuronal process outgrowth and in axon stability. Phenotypic analysis of mutant worms and genetic interaction studies with worm homologs of N-WASP, WAVE, and Ena/VASP suggest that septins function with regulators of the actin cytoskeleton. We hypothesize that septins promote assembly of the axonal actin cytoskeleton into the branched filaments found in lamellipodia that are necessary for robust axon outgrowth, and that in the absence of septin function, formation of filopodia is favored.

1914/B293
Septins Regulate the Formation and Maturation of Axonal Filopodia to Collateral Branches.
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Development of a functional nervous system requires the formation of correct patterns of innervation, which are largely shaped by the collateral and terminal branching of axons. Collateral branches are formed from axonal filopodia, which arise from pre-existing F-actin patches within the axon shaft. Subsequently, axonal microtubules are guided and targeted into filopodia, which mature to collateral branches. The molecular mechanisms that regulate the spatial decisions of the actin and microtubule cytoskeletons during this process are poorly understood. Here, we present data that suggest that formation of collateral branches may be spatially regulated by septins, a novel family of GTP-binding proteins that associate with actin and microtubules. Using embryonic sensory neurons (chicken dorsal root ganglia) as a model system, we found that septin 7 (SEPT7) overlaps with both F-actin patches and microtubules in the axonal shaft of neurons. By platinum replica electron microscopy (EM) and immune-EM, SEPT7 appeared as granular rod-shaped structures tightly associated with axonal microtubules. Significantly, we observed that SEPT7 concentrates at the base of axonal filopodia. Over-expression of SEPT7-GFP increased the life-time of axonal filopodia, but not the rate of their formation, leading to an overall increase in the number of filopodia. Presence of tubulin within the majority of SEPT7-GFP-containing filopodia suggests that these structures were in transition to becoming axonal branches. In contrast to the role of SEPT7 in the maturation of axonal filopodia, overexpression of SEPT6-GFP and stabilization of septins with the drug forchlorfenuron increased the rate of filopodia formation. These data suggest that septins 6 and 7 may differentially regulate the actin and microtubule cytoskeleton during the formation and maturation of axonal shaft filopodia to collateral branches, respectively.

Cell Migration III (1915 – 1942)

1915/B294 ABSTRACT WITHDRAWN

1916/B295
SSXs Regulate Cancer Invasion via Rac1-Myosin Pathway and MMP-1 Signaling.
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The SSX genes were initially identified as fusion partners to the SS18 gene in human synovial sarcomas carrying a recurrent t(X;18)(p11.2;q11.2) chromosomal translocation. Besides adult human testis, SSX genes were expressed at varying frequencies in a number of malignancies thereby categorized as cancer/testis antigens. We previously reported that SSXs were overexpressed in human soft tissue tumors, and positively correlated with clinical stage. In order to examine the biological function of SSX, we made stable transfectants with wild type SSX using human fibrosarcoma cell line, HT1080, which endogenously expressed SSX1 in high level. The transfectants increased motility and invasiveness using Boyden chamber assay, promoted colony formation in soft agar and lung metastasis in nude mice. By contrast, the lowering of the endogenous expression of SSX1 in HT1080 cells by the treatment with specific siRNA markedly decreased membrane ruffling, chemotaxis, invasiveness and 3D growth in collagen gel but did not affect cell proliferation in the 2D culture. Moreover, SSX1 deficient HT1080 cells showed decreased Rac1-activity and myosin light chain phosphorylation. Overexpression of SSX increased the expression level and activity of MMP-1, and knockdown of SSX decreased MMP-1 expression. A specific MMP-1 inhibitor (GM6001) decreased invasion, but did not affect SSX-induced Rac1-activity and myosin light chain phosphorylation. We identified several binding proteins of SSX, including Histone H1 and PARP-1 using immunoprecipitation and LC/MS/MS. We further confirmed that SSX could bind the promoter region of MMP-1 using ChIP assay. Wrapped liposome containing SSX siRNA effectively inhibited both primary tumor growth and lung metastasis in xenograft model. Collectively, these data suggested that SSXs would be novel proteins regulate cancer invasion via Rac1-myosin pathway and MMP-1 signaling, and potential molecular target(s) under clinical setting.

1917/B296
Rapamycin Suppresses Metastatic Capability of Sarcomatous Cholangiocarcinoma by Blocking STAT3 Signaling.
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Cholangiocarcinoma (CC) is a malignant epithelium neoplasm which originates from the bile epithelium. A lipophilic macrolide antibiotic, rapamycin, is known to inhibit mammalian target of rapamycin (mTOR) which plays a key role in migration, invasion, protein translation and cellular growth and was suggested as one of the effective drug candidates to target some cancer types. However, the underlying molecular mechanism remains unclear. mTOR was found to phosphorylate STAT3 in some cell lines and the activation of STAT3 was critical for cholangiocarcinogenesis where matrix metalloprotease 2 (MMP2) and twist1, regulated by STAT3, were critical for epithelial-mesenchamal transitions (EMT). The regulation of transcriptional activity of STAT3 was found to be important in the antitumor effects of rapamycin on a sarcomatous CC cell line, SCK. Rapamycin dramatically blocked the invasion and migration ability of SCK, mediating the down-regulation of p-STAT3 (S727). The transcription and translation levels of MMP 2 and twist 1 were decreased in the dose dependent manner due to the reduction of STAT3 transcriptional activity. To investigate that the regulation of transcriptional activity of STAT3 was important for rapamycin inhibition effects on sarcomatoid SCK, we compared the rapamycin sensitivity of STAT3. The expression levels of MMP2 and Twist1 were decreased in SCK transfected with STAT3 S727A compared to STAT3 WT. In addition, as for STAT3 S727A negative mutant, the expression levels of MMP2 and Twist1 were reduced less than those of STAT3 WT upon the rapamycin treatment. The migration and invasion assay demonstrated that SCK transfected transiently with the negative mutant form decreased the motility and was more insensitive to rapamycin relative compared to STAT3 WT. Taken together, our results indicate that rapamycin can suppress tumor malignancy of CC by inhibiting the STAT3 signaling pathway.

1918/B297
Selective Inhibition of Prostate and Breast Cancer Migration and Proliferation by a Boric Acid Derivative, Phenylboronic Acid.
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While most cancer therapies focus on controlling tumorigenic cell proliferation, few treatments are available to clinically treat metastasis, and none of them are curative. An additional problem with many cancer therapeutics is an inability to distinguish between targeting healthy and tumorigenic cells. Therefore, we sought to identify novel compounds that selectively target the metastatic stage of cancer. A promising anticancer compound, boric acid (BA), decreases proliferation and migration of one prostate cancer cell line at a concentration of 1mM. To identify a more potent derivative, phenylboronic acid (PBA) was selected for further studies. The In Vitro effects of BA and PBA on prostate cancer (DU-145, PC-3) and breast cancer (ZR-75-1) cell lines were compared to a normal prostate cell line (RWPE-1) or a non-tumorigenic mammary epithelium line (MCF-10A), respectively, using migration, adhesion, and proliferation assays. Treatment up to 24 hours with BA did not inhibit migration on fibronectin in any cancer cell line with concentrations as high as 500µM. However, treatment over the same time course with PBA significantly inhibited DU-145, PC-3, and ZR-75-1 cell migration, in a dose dependent manner, at concentrations as low as 1µM. The same concentrations of BA and PBA that inhibited prostate and breast cancer chemotaxis did not decrease migration of the corresponding normal cell lines. Furthermore, no concentration of BA or PBA caused a significant decrease in adhesion to or spreading on fibronectin in any cell line, and the compounds were noncytotoxic at 24 hours at 1mM. With long term treatment, the compounds decreased proliferation in the cancer lines but not the normal cells. Preliminary results suggest that BA and PBA may inhibit cancer cell migration and proliferation by decreasing activation of focal adhesion kinase (FAK), causing changes in downstream signaling of proteins involved in regulating the actin cytoskeleton, including Src, RhoA, Cdc42, Rac1, and calpain. The selectivity of PBA for tumorigenic cells, and its ability to elicit an anti-migratory response at concentrations significantly lower than BA, makes it a promising novel compound for the treatment of cancer.

1919/B298
MicroRNA-138 Regulates Cell Migration and Invasion by Targeting the Rho GTPase Signaling Cascade in Tongue Squamous Cell Carcinoma.
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Tumor metastasis is the dominant cause of death in cancer patients, including patients with oral tongue squamous cell carcinoma (TSCC). Previously, we reported that reduced miR-138 level is correlated with enhanced metastatic potential in TSCC cells. Here, we demonstrate that miR-138 suppresses cell migration and invasion by regulating two key genes in the Rho GTPase signaling cascade: RhoC and ROCK2. Direct targeting of miR-138 to specific sequences located in the 3'-untranslated regions of both RhoC and ROCK2 mRNAs were confirmed using luciferase reporter gene assays. Ectopic transfection of miR-138 reduced the expression of both RhoC and ROCK2 in TSCC cells. This led to the reorganization of the stress fibers and the subsequent cell morphology change to a round bleb-like shape, as well as the suppression in cell migration and invasion. In contrast, the knockdown of miR-138 enhanced the expression of RhoC and ROCK2 and resulted in an altered, elongated morphology, and enhanced cell migration and invasion. Thus, our results indicate that miR-138 plays an important role in TSCC cell migration and invasion by concurrently targeting RhoC and ROCK2, and may serve as a novel therapeutic target for TSCC patients at risk of metastatic disease.

1920/B299
The Role of the Hippo Pathway in Promoting Cancer Cell Migration and Metastasis.
In Drosophila, the Hippo pathway plays an essential role in regulating cell proliferation and apoptosis. Within the mammalian counterparts of this pathway is the coactivator YAP (Yes-associated protein), whose direct phosphorylation by the activated Hippo pathway leads to its inhibition and, therefore, the translocation of YAP from the nucleus to the cytoplasm where it is sequestered by 14-3-3. Genetic mutations in this pathway lead to the activation of YAP and the transcription of genes that promote cell proliferation and survival. Recently, it has been shown that YAP overexpression in MCF10A human mammary epithelial cells leads to an epithelial-mesenchymal transition (EMT) as well as soft agar colony formation. Based on these and other previous experiments, we sought to elucidate the mechanism and role of YAP in promoting anchorage independent growth and migration in metastasis. To better understand the mechanism behind YAP induced cell migration and survival, we have tested the phosphorylation levels of known regulators of proliferation and cell migration in mammals. There are significant differences in the phosphorylation states of Erk (increase), Paxillin (decrease) and p130Cas (decrease) when comparing adherent 5SA mutant, constitutively activated YAP, versus vector control MCF10A cells. We also show that the YAP 5SA mutant expression caused an increase in Erk activation in suspension culture, which suggests a possible mechanism for anchorage independent growth and survival. Finally, the addition of the MEK inhibitor, PD98059, eliminates the increased migratory phenotype of the YAP 5SA cells in 2D culture. Through these studies, we have concluded that YAP overexpression significantly affects Erk activation through a Ras dependent mechanism leading to increased migration of the cancer cell.

1921/B300
Secreted Heat Shock Protein 90 (Hsp90): A Critical Pro-motility Factor during Wound Healing.
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The care for human skin wounds, stasis and pressure ulcers, diabetic ulcers, burns and others, costs the United States ~$11 billion/year. For example, 71,000 Americans underwent lower extremity amputations in 2005 (the latest available data) due to non-healing diabetic ulcers, in which just a single surgical procedure and hospitalization alone costs $27,000 to $65,000. The currently available treatments for non-healing wounds, such as RegranexTM (PDGF-BB), are only moderately effective and are very expensive. Thus, there is a pressing need to develop new and cost-effective wound healing agent(s). Until recently, heat shock protein-90 (Hsp90) has been mostly known as an ATP-dependent intracellular chaperon protein with more than 100 client targets inside the cell. However, studies of past five years have unveiled a surprising need for cells to secrete Hsp90a to the extracellular environment under pathological conditions, such as in a wounded tissue. Our laboratory has reported that both epidermal keratinocytes and dermal fibroblasts rapidly secrete Hsp90a under hypoxia or TGFa presence, two acute signals when skin is wounded. Outside the cell, secreted Hsp90a promotes cell motility through binding to the LDL-Receptor Related Protein-1 (LRP1) receptor. The pro-motility activity resides between the Linker Region and the Middle Domain of Hsp90a, but is independently of its N-terminal ATPase and C-terminal dimerization domains. More intriguingly, unlike conventional growth factors, Hsp90a-induced cell migration cannot be blocked by anti-motility effect of TGFb, which is abundantly present in the wound bed. This finding explains how dermal cells (fibroblasts and endothelial cells) migrate into the TGFb-rich environment in the wound. Most importantly, topical application of purified Hsp90a significantly accelerated the wound-healing rate in mice. In parallel, RegranexTM (FDA-approved PDGF-BB to treat diabetic ulcers) showed much less effect. Thus, we propose here to develop Hsp90 into a novel, effective and low cost wound-healing agent.

1922/B301
Inhibition of p70S6 Kinase Prevents Invasive Migration of Brain Tumor Cells.
Glioblastoma multiforme (GBM) are extremely aggressive, malignant tumors that develop in the brain. GBMs often migrate away from the initial tumor and spread to distant regions of the brain. The clinical management of GBMs consists of surgical resection followed by radiotherapy and chemotherapy. Activation of several signaling pathways has been associated with the invasive capacity of GBM cells. Activity of the Akt/mTOR pathway is exceptionally high in glioblastoma multiforme and participates in the regulation of invasive migration. A downstream effector of Akt and mTOR, p70S6 kinase (p70S6K) demonstrates a high amount of activity in GBM tumor samples and GBM tissue culture cell lines. Inactivation of p70S6K attenuates GBM cell growth and may inhibit cell migration. Therefore, we tested the potential of Ro 31-8220 a p70S6K inhibitor in preventing GBM cell migration. Human GBM cell lines (U-251MG) exposed to Ro 31-8220 (5 µM) demonstrated a 70% decrease in the invasive capacity of these cells in transwell migration assays. In order to determine the specific mechanism of Ro 31-8220 inhibition in these cells the adhesive capacity, ability to migrate and the production of proteases was tested. Ro 31-8220 does not prevent U-251MG cell adhesion. Using video time-lapse microscopy to determine the migration capacity of U-251MG cells, Ro 31-8220 treated cells demonstrated a 10% reduction in cell migration after 7 days. Since exposing cells to Ro 31-8220 did not prevent cell adhesion and demonstrated only a minimal inhibitory effect on cell migration, the secretion of degradative proteases was examined. Pretreatment of U-251MG cells with Ro 31-8220 decreased the production of MMP-9 and uPA proteins as well as uPA protease activity. Utilizing siRNA directed against p70S6K decreased the protease activity of U-251MG cells in culture. Taken together these data suggest that p70S6K positively regulates GBM cell invasion by promoting protease production.

1923/B302
Asymmetry of PI3K Signaling Dynamics Coincides with Directional Fidelity of Fibroblast Chemotaxis.
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Fibroblast chemotaxis stimulated by platelet-derived growth factor (PDGF) plays a pivotal role in the proliferative phase of cutaneous wound healing. PDGF receptor-mediated signaling processes that preside over cell motility in this context include several phosphoinositide 3-kinase (PI3K)-dependent pathways and signaling through Rho-family GTPases. Although it is well established that PDGF gradients elicit asymmetric patterns of PI3K signaling in fibroblasts, we are presently concerned with how the spatiotemporal dynamics of PI3K signaling, imaged in cells over extended periods of time, are related to the directional persistence of PDGF-stimulated chemotaxis. To address this question, we apply Signaling Vector Analysis (SVA), a suite of analytical methods for relating parallel live-cell microscopy measurements of cell migration dynamics and the intracellular signaling processes that govern them.

1924/B303
The Sca1/RasGEF/PP2A Signaling Complex Controls the Ras-TORC2-Akt/PKB Pathway and Directed Cell Migration.
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Ras plays a central role in Dictyostelium chemotaxis, acting, in part, through the regulation of phosphatidylinositol-3 kinase (PI3K) and target of rapamycin complex 2 (TORC2). Ras activation appears to lie immediately downstream from receptors and heterotrimeric G proteins and evidence suggests that Ras is part of the gradient sensing machinery. The present study was undertaken to identify new regulators of Ras signaling in Dictyostelium chemotaxis and to better...
understand the mechanisms implicated in the spatiotemporal control of Ras activity. Using a proteomic approach, we identified a stable protein complex that contains the Aimless RasGEF, another RasGEF, RasGEFH, a previously uncharacterized protein PHR, and the protein phosphatase 2A (PP2A), brought together by a scaffold designated Sca1. Whereas disruption of phr or gefH produced marginal effects on chemotaxis, cells lacking Aimless or Sca1 lack chemoattractant-induced activation of the Dictyostelium H-Ras orthologue RasC and adenyl cyclase, display reduced PKB activity and decreased polymerization of F-actin, and exhibit defects in cell motility and chemotaxis. We show that the Sca1-scaffolded signaling complex regulates PKB as well as the PKB-related protein PKBR1, by controlling the RasC-dependent activation of TORC2, but not that of PI3K, and that the Sca1 complex is recruited to the plasma membrane upon uniform chemoattractant stimulation and to the leading edge of chemotaxing cells. Moreover, we show that Sca1 undergoes transient, chemoattractant-induced PKB/PKBR1-promoted phosphorylation and that PKB/PKBR1 negatively regulate the localization of the Sca1 complex as well as RasC activity. Finally, preliminary data suggest that the presence of PP2A in the complex is necessary for the recruitment of the Sca1 complex to the plasma membrane and the RasC-promoted activation of PKB. We propose that the Sca1/RasGEF/PP2A signaling complex controls the activation of the RasC-TORC2-PKB/PKBR1 pathway, and undergoes PKB/PKBR1-promoted negative feedback regulation that most likely includes the direct phosphorylation of Sca1 and inhibition of its membrane translocation.

1925/B304
mTORC2-Mediated AC9 Activation Is Required for Chemoattractant-Induced cAMP Production and Chemotaxis in Neutrophils.
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Neutrophils and Dictyostelium use conserved signal transduction pathways to decipher chemoattractant gradients and migrate directionally. In both cell types, addition of chemoattractants induces transient increases in intracellular cAMP. In Dictyostelium, cAMP synthesis is mediated by the adenyl cyclase ACA, which requires inputs from heterotrimeric G proteins, phosphoinositide-3 kinase (PI3K), and the target of rapamycin complex 2 (TORC2). In this organism, cAMP regulates group migration and lateral pseudopod formation. Here, we set out to define the mechanisms by which chemoattractants increase cAMP levels in neutrophils and determine the role of cAMP during neutrophil chemotaxis. We previously showed that four of the nine mammalian G protein-coupled adenyl cyclases are expressed in neutrophils, with AC9 being predominant. We now show that RNAi-mediated knockdown (KD) of AC9 in neutrophil-like cells decreases basal cAMP levels and inhibits chemoattractant-induced cAMP production. Moreover, while AC9 KD cells can sense chemoattractant gradients by polarizing and correctly orienting, they cannot translate this information into robust migration. on the other hand, over-expression of AC9 leads to higher basal cAMP levels and triggers the formation of abnormal long cellular tails that are highly enrich in P-Myosin II. While the inhibition of PI3K activity does not alter the ability of chemoattractants to induce cAMP production, the inhibition of mTORC2 activity (achieved via KD of Rictor) severely inhibits the chemoattractant-induced cAMP production. Compared to WT cells, Rictor KD cells show a significant loss in cell polarity and cannot migrate to a source of chemoattractant, much like Dictyostelium cells lacking components of TORC2. In conclusion, we find that signals regulating the chemoattractant-mediated cAMP production are highly conserved between Dictyostelium and human neutrophils. In both systems, TORC2 appears to be a key regulator of cell polarity and migration. On the other hand, intracellular cAMP levels seem to specifically regulate back retraction during migration. We envision that conserved downstream signals mediate these effects.

1926/B305
A Novel Role for Calcium-Independent Phospholipase A2 in the Regulation of Src Trafficking and Microglia Chemotaxis.
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Microglia are the immune effector cells in the central nervous system. Microglia in the brain participate in the response to brain injuries and diseases in which their activation, migration, and proliferation are known to play crucial roles. We examined the role of Ca++-independent phospholipase 2 (iPLA2) for the regulation of microglia chemotaxis toward ADP. As shown in Dictyostelium, low concentration of LY294002 and BEL, an iPLA2-specific inhibitor, exerted a synergistic inhibition on microglia chemotaxis. Careful dissection of signaling pathways involved in the regulation of microglia chemotaxis revealed that trafficking of c-Src to the plasma membrane is significantly impaired in cells treated with BEL or iPLA2 knock-down cells. Immunostaining of c-Src showed that c-Src was accumulated in the trans-Golgi area that can be co-stained with WGA in BEL-treated cells, indicating that post-Golgi trafficking of Src to the membrane is significantly impaired. iPLA2 activity appears to be specifically required for the trafficking of c-Src or K-Ras since VSV-G trafficking to the membrane was not affected by BEL. These results suggest the participation of calcium-independent phospholipase A2 in membrane trafficking and in the regulation of chemotaxis through the secretory pathway.

1927/B306
The Non-receptor Tyrosine Kinase Lyn Is Required for De-Adhesion during Neutrophil Chemotaxis.
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Chemotaxis, the directed movement of cells in a gradient of chemoattractant, allows neutrophils to migrate to sites of inflammation and infection. Despite recent advances in our understanding of biochemical regulation of neutrophil polarity, little is known about the molecular program controlling neutrophil - extracellular matrix (ECM) interactions during chemotaxis. By using genetically manipulatable neutrophil-like HL-60 cells, we identified the non-receptor tyrosine kinase Lyn, a member of the Src family kinases, as a key regulator of neutrophil de-adhesion. In response to chemoattractants, Lyn is activated and recruited to the leading edge of polarizing neutrophils. Perturbations of the Lyn function in neutrophils by the use of inhibitory peptides, dominant negative mutants or siRNA-mediated knockdown impair directional migration and prevent cells from de-attaching their trailing edges. Interestingly, inhibition/depletion of Lyn fails to affect Myosin II-mediated contractility of cells at the back and the sides of neutrophils, suggesting that Lyn mediates neutrophil de-adhesion independently of Myosin II-generated cytoskeletal forces. At the molecular level, Lyn depletion causes activated beta2-integrin to upregulate on the neutrophils’ surface and talin to re-distribute from the leading edge to the trailing edge, leading to enhanced cellular adhesion to the ECM substrate at the rear of the cells. Taken together, these results suggest that chemoattractants induce neutrophils to activate Lyn, which negatively modulates integrin activity to facilitate neutrophil de-adhesion and to promote persistent directional migration. Therefore, our findings have revealed a novel signaling mechanism that governs de-adhesion during movement of neutrophils, and possibly other amoeboid cells.

1928/B307
Asymmetric PI3K Signaling Predicts Persistence of Adhesion-driven Cell Migration.
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There is ample evidence that aspects of cell migration are stochastic; how else could it exhibit directional persistence, over time scales much longer than typical signal transduction processes, punctuated by abrupt changes in direction? To better understand how those behaviors are affected by intracellular signaling pathways, we have developed new analytical methods for relating parallel live-cell microscopy measurements of cell migration dynamics and the
intracellular signaling processes that govern them. Analysis of phosphoinositide 3-kinase (PI3K) signaling in fibroblasts randomly migrating on fibronectin and surface-grafted RGD peptides reveals that hot spots of intense signaling coincide with localized cell protrusion. Loss of directional persistence coincides with instability of the asymmetric signaling pattern, indicative of a mechanism by which changes in a cell’s direction of migration are determined by a fragile balance of relatively rapid intracellular signaling processes.

1929/B308
Regulation of Keratinocyte Attachment, Invasion and Differentiation by the hScrib and hDlg Cell Polarity Regulators.
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Previous studies have shown essential roles for the hScrib/hDlg complex in the regulation of cell polarity, attachment and migration in canine kidney and human breast epithelial cells. However little is known about the role of these proteins in the context of a differentiating epithelium. This is particularly relevant since Human Papillomaviruses (HPVs) target both of these proteins during the induction of cervical malignancy. Therefore we have been interested in analyzing the respective functions of hScrib and hDlg in human keratinocytes, the natural target cells of the virus. Induction of MAPK signaling, results in a dramatic accumulation of both proteins at sites of cell contact. Whilst there is a degree of interdependency upon the correct localization of hDlg and hScrib under non-stressed conditions, this interdependency is lost upon activation of MAPK. Based on this we wished to determine whether loss of either protein would have similar phenotypes in a variety of assays designed to investigate cell attachment and migration. Interestingly, loss of hDlg expression results in a greatly decreased rate of cell attachment, which is in marked contrast to hScrib knockdown, where the cells attach with wild type efficiency. Likewise, in cell migration, Dlg -/- cells are weakly invasive, whilst Scrib -/- cells reveal an enhanced invasive potential. These studies demonstrate quite distinct functions for hDlg and hScrib in the context of human keratinocytes, and suggest that targeting of hScrib by HPV is likely to result in a more invasive phenotype than the viral targeting of hDlg. Current studies are now aimed at investigating the respective roles of hDlg and HaScrib in keratinocyte differentiation.

1930/B309
The Rho Family GEF Asef2 Activates Rac to Modulate Adhesion and Actin Dynamics and thereby Regulate Cell Migration.
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The Rho family of small GTPases, which includes Rho, Rac and Cdc42, are critical regulators of the actin cytoskeleton. Activation of these small GTPases is tightly controlled by guanine nucleotide exchange factors (GEFs), which promote the exchange of bound GDP for GTP. Once activated, the GTPases can convert upstream molecular signals into coordinated actin cytoskeleton rearrangements by modulating the activity of downstream effectors, which can ultimately contribute to the regulation of cell migration and adhesion dynamics. Asef2 is a recently identified Rho family GEF that has been implicated in modulation of actin dynamics, but its function in cell migration and adhesion dynamics is not well understood. In this study, we show Asef2 is an important regulator of migration and adhesion assembly and disassembly (turnover). Knockdown of endogenous Asef2 expression using siRNA dramatically decreases the migration velocity and slows the rate of adhesion turnover of HT1080 cells. Stable expression of low levels of GFP-Asef2 promotes the rapid turnover of adhesions and cell migration. Asef2 significantly enhances both Rac1 and Cdc42 activity in HT1080 cells, but only Rac1 is critical for the Asef2 promoted increase in migration and adhesion turnover. Phosphoinositide 3-kinase (PI3K) and the serine/threonine kinase Akt are also essential for the Asef2-mediated effects on these processes. Asef2 signaling leads to an overall decrease in Rho activity by a mechanism that is dependent on Rac, PI3K, and Akt. This previously unknown mechanism, induced by Asef2, is critical for
stimulating migration and adhesion dynamics. Our results reveal an important new role for Asef2 in promoting migration and rapid adhesion turnover by coordinately regulating the activities of Rho family GTPases. Our working model is that Asef2 signaling promotes a local increase in Rac activity and a decrease in the amount of active Rho, which stimulates adhesion turnover and thereby enhances cell migration.

1931/B310
The Cytohesin-binding Protein, IPCEF1, Interacts with RhoA.
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The ARF family of small GTPases are regulators of vesicular trafficking, migration and actin rearrangements. Activation of ARF6 by the GEF ARNO/cytohesin 2 increases the migration of epithelial cells. ARF6 activation is also required for epithelial migration in response to the growth factor HGF. We have now found that protein-protein interaction domain in ARNO, the coiled-coil domain, is required for the increased motility seen in ARNO expressing cells. Several scaffolding proteins bind to this domain. IPCEF1 is one of these cytohesin-binding proteins. We show that knockdown of IPCEF inhibits the migration of MDCK cells in a wound healing assay. The PH domain of IPCEF is homologous to several other PH domains that bind to members of the Rho family in a GTP dependent manner. The Rho family of small GTPases are established regulators of cell motility via actin cytoskeleton remodulati on. We have found that IPCEF binds to active RhoA but not to either active Rac or active cdc42. Since the constitutively active mutants of RhoA inhibit migration of MDCK cells in response to HGF (Ridley, 1995), we suspect that this interaction negatively regulates the activation of ARF6 by HGF. Therefore, we are evaluating how constitutively active RhoA Q63L and dominant negative RhoA T19N affect the activation of ARF6 by HGF using GST-GGA3 pulldown studies.

1932/B311
Regulation of ROCK by its C-terminus.
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ROCK or Rho kinase is a ser/thr kinase that regulates cell adhesion, migration, invasion, cytokinesis, apoptosis, and oncogenic transformation. ROCK induces these effects primarily by phosphorylation of different substrates; however, the mechanisms that determine which downstream signaling pathways are activated in specific biological contexts are not presently understood. Based on the range of biological functions for ROCK, it is reasonable to expect that specification of downstream signaling events is regulated by cell type, subcellular localization, and extracellular stimuli. ROCK contains an N terminal kinase domain, a central coiled coil domain containing RBD (Rho binding domain) and a PH (Pleckstrin homology) domain at its C terminus. PH domains are known to bind to lipids and proteins. It is possible that different motifs present in ROCK might fine tune its localization in different subcellular compartments of the cell. Using live cell microscopy we found that full length GFP ROCK2 is transiently localized in membrane ruffles. Extracellular stimuli, such as SDF-1, promote the localization of ROCK 2 in membrane ruffles. The RBD and the PH domain have both been hypothesized to regulate ROCK localization. We have found that deletion of the PH domain prevents the localization of ROCK at the membrane indicating that PH domain is required for membrane localization of ROCK2. In addition, prenylated active Rho efficiently translocates ROCK to the membrane. Based upon our data we hypothesize that, the RBD and the PH domain receive localization signals and thus the C terminus of ROCK integrates and fine tunes its localization.

1933/B312
The Adaptor Protein APPL1 Regulates Cell Migration and Adhesion Dynamics through Akt Signaling.
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Even though kinases and phosphatases receive much of the attention, adaptor proteins are emerging as important regulators of cell migration. They bring together key signaling components to coordinate regulatory processes underlying migration, such as actin reorganization and adhesion turnover. Here, we show for the first time that the adaptor protein containing PH domain, PTB domain, and leucine zipper motif 1 (APPL1) is a critical regulator of cell migration. Expression of GFP-APPL1 in HT1080 cells decreases their migration speed by approximately 2-fold while knockdown of endogenous APPL1 using siRNA significantly increases their migration speed. Deletion of the phosphotyrosine binding (PTB) domain of GFP-APPL1, which interacts with the serine/threonine kinase Akt, abrogates the APPL1-mediated effects on migration, leading us to hypothesize that this interaction is important for the function of APPL1 in this capacity. To test this, we expressed various Akt mutants with APPL1 and examined the effect on migration. Co-expression of GFP-APPL1 with constitutively active Akt (CA-Akt) negates the CA-Akt promoted increase in migration, while co-expression with a dominant negative Akt (DN-Akt) shows no further decrease in migration compared to DN-Akt alone. Moreover, GFP-APPL1 expression decreases the amount of active Akt in HT1080 cells, suggesting APPL1 inhibits migration by interfering with Akt function. Interestingly, HT1080 cells expressing GFP-APPL1 have an increased number of large adhesions and a decreased number of small peripheral adhesions compared with control cells. Since the assembly and disassembly of adhesions (adhesion turnover) is critical for migration, we thought APPL1 might regulate migration by altering adhesion turnover. Indeed, expression of GFP-APPL1 significantly slowed adhesion turnover, suggesting APPL1 is a regulator of this process. Collectively, our results suggest a new role for APPL1 in the regulation of cell migration and adhesion dynamics. In addition, this work provides new and exciting insight into how adaptor proteins can coordinate signaling pathways to modulate adhesion turnover and thereby regulate cell migration.

1934/B313
**Mammalian Target of Rapamycin Complex 2 and P-Rex1 Modulates Akt1 Activation and Regulates Cancer Cell Migration.**

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The mammalian target of rapamycin (mTOR), which is a highly conserved serine/threonine kinase, plays various roles in growth, proliferation, and migration. Here, we showed that molecular complex formation of mTORC2/P-Rex1/Akt1 is necessary for insulin-like growth factor-1 (IGF-1)-induced migration of human ovarian cancer (SKOV-3) cells. IGF-1-induced SKOV-3 cell migration was completely blocked by phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) or Akt inhibitor (SH-5). However, inhibition of ERK or mTORC1 did not affect IGF-1-induced SKOV-3 cell migration. Furthermore, knock-down of Akt1 completely blocked IGF-1-induced SKOV-3 cell migration whereas knock-down of Akt2 was not effective. Silencing of mTOR or Rictor abolished IGF-1-induced SKOV-3 cell migration. However, knock-down of Raptor did not affect IGF-1-induced SKOV-3 cell migration. Akt1 was preferentially associated with Rictor and expression of Rictor strongly enhanced IGF-1-induced phosphorylation of Akt1 but not Akt2. In addition, P-Rex1 was preferentially associated with Akt1 and expression of P-Rex1 significantly promoted IGF-1-induced phosphorylation of Akt1 but not Akt2. Finally, silencing of P-Rex1 resulted in the attenuation of IGF-1-induced Akt phosphorylation, migration, as well as Rac activation. Given these results, we suggest that mTORC2/P-Rex1/Akt1 axis plays pivotal roles in IGF-1-induced cancer cell migration.

1935/B314
**PKA-mediated Phosphorylation of VASP at Ser 157 and Activation of the Small GTPase Rap1 Contribute to IGF-I-induced Cell Migration.**

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Ample studies reported that the activation of the insulin-like growth factor type I receptor (IGF-IR) can promote the migration of cells, including breast cancer cells. The objective of our research is to reveal the intracellular network by which IGF-IR regulates cell migration and that remains incompletely understood. We demonstrated previously that the formation of protrusions containing actin-fascin microspikes in MCF-7 human breast cancer cells is a reliable read-out for the initiation of breast cancer cell migration by IGF-I. Here, we found that the IGF-I-induced movement of MCF-7-derived cells was accompanied by the increased phosphorylation of vasodilator-stimulated phosphoprotein VASP at the preferred Protein Kinase a (PKA) phosphorylation site Ser 157. The intrinsic tyrosine kinase activity of the receptor was required for VASP phosphorylation as blocking endogenous activity of the IGF-IR by over-expression of its dead-kinase mutant prevented phosphorylation of VASP-Ser 157 in response to IGF-I. Pretreatment of cells with PKA inhibitors, H-89 and myrPKI also decreased IGF-I-induced phosphorylation of VASP-Ser157 in a time-course experiments confirming that phosphorylation of VASP downstream of the IGF-IR is indeed regulated by PKA. Furthermore, inhibition of the IGF-IR-PKA signaling blocked the IGF-I-induced formation of motile protrusion containing actin-fascin, indicating the importance of the IGF-IR-PKA pathway for the initiation of breast cancer cell movement. Interestingly, phosphorylation of VASP-Ser 157 induced by agonists of cAMP/PKA signaling, Sp-cAMP and forskolin did not cause cell movement, suggesting that apart from affecting VASP, the IGF-IR-PKA pathway must control additional signaling contributing to cell movement by IGF-I. We propose that small GTPase Rap1 is an important player downstream of IGF-IR-PKA, because (i) inhibition of either IGF-IR (by dead-kinase mutant) or PKA (by H-89) blocked Rap1 activation by IGF-I and (ii) down-regulation of C3G, a Rap1 guanine nucleotide exchange factor (GEF), by C3G siRNA inhibited actin-fascin microspikes in IGF-I-stimulated cells.

Melanin Concentrating-Hormone Facilitates the Migration of Cultured 3T3-L1 Pre-adipocytes.
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Melanin-concentrating hormone (MCH) is an appetite-stimulating hormone, which binds G protein-coupled receptors (GPCRs) found in neuronal, pancreatic, and adipose tissues. The broad distribution range of its receptors suggests that MCH might help regulate the whole-body response to food. We recently discovered that in addition to eliciting a MAPK signal in 3T3-L1 pre-adipocytes, MCH also mediates rearrangements of the actin cytoskeleton, leading to a reversible change in cell morphology; however the physiological significance of this change is as yet unknown. The aim of this study was to determine whether MCH-mediated actin rearrangements result in altered pre-adipocyte migration. 3T3-L1 pre-adipocytes, endogenously expressing MCH receptors, were grown to confluency, wounded, and then treated with MCH. At hour-long time points, the wound was measured and compared to the size of the initial wound. The percent of closure was measured in both the presence and absence of MCH. When cell migration experiments were performed in the absence of serum, there was little difference between MCH treated and control cells for as long as 12 hours. However, in the presence of serum, MCH caused cells to migrate into the wound progressively faster. After six hours, the hormone-treated wounds had closed to 39.3±6.7% of the initial wound size versus the untreated wounds which had only closed to 49.5±8.7% of the initial size (significant to the 98th percentile). This novel finding suggests that while MCH does not influence pre-adipocyte differentiation, it could impact the overall development of adipose tissue in vivo.

RLIP76 Recruits The Arf Guanine Exchange Factor ARNO To Activated R-Ras To Induce Activation Of Arf6 Leading To Cell Motility.
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We previously identified Ral-binding protein 1 (RalBP1/RLIP76) as a selective effector of the Ras family GTPase R-Ras, and found that RLIP76 is required for R-Ras-dependent activation of the small GTPases Arf6 and Rac1 leading to cell spreading and migration. We further found that RLIP76 binds cytohesin-2/ARNO, a guanine nucleotide exchange factor which activates Arf1 and Arf6 and regulates cell motility. We now find that RLIP76 recruits ARNO to activated R-Ras to induce R-Ras-dependent signaling leading to enhancement of cell motility. ARNO forms a tri-molecular protein complex in cells with activated R-Ras and RLIP76. ARNO binding to R-Ras requires GTP loading of R-Ras and the presence of RLIP76 in the complex, suggesting that RLIP76 acts as a scaffold protein to connect ARNO to activated R-Ras in cells. We mapped the R-Ras binding region to the RLIP76 RhoGAP domain, whereas ARNO binds the RLIP76 N-terminus. Using a knockdown/reconstitution approach, we find that the N-terminal domain of RLIP76 is required for adhesion-induced Arf6 and Rac1 activation, cell spreading and cell motility. Over-expression of RLIP76 lacking the N-terminal domain (RLIP76ΔN) or of the N-terminal domain alone prevents ARNO association with R-Ras, and blocks adhesion-induced Arf6 and Rac1 activation. Furthermore, RLIP76ΔN or the RLIP76 N-terminal domain blocks cell spreading and migration. Thus, RLIP76 provides a critical role in cell motility by physically linking Arf6 activation machinery to the pro-migratory protein, R-Ras.

1938/B317
High Mobility Group Box 1 (HMGB1) Inhibits Enterocyte Migration via Activation of Toll Like Receptor 4.
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Necrotizing enterocolitis (NEC) is the most common cause of gastrointestinal-related mortality in neonates, and is characterized by increased enterocyte Toll-like-receptor 4 (TLR4) signaling and impaired epithelial barrier integrity. The endogenous ligands for TLR4 leading to barrier injury in the pathogenesis of NEC remain unknown. High mobility group box-1 (HMGB1) is a DNA-binding protein that is released from injured cells and activated macrophages during inflammation, and induces organ injury through ill-defined mechanisms. We now hypothesize that HMGB1 participates in the pathogenesis of NEC through TLR4 activation on enterocytes and the inhibition of mucosal healing. In support of this hypothesis, we now demonstrate that murine and human NEC are associated with increased intestinal HMGB1 expression, and that HMGB1 inhibits enterocyte migration In Vitro and In Vivo in a TLR4-dependent manner. In seeking to understand the mechanisms involved, HMGB1 signaling via TLR4 caused increased RhoA activation in enterocytes, leading to increased phosphorylation of focal adhesion kinase and phosphorylation of the actin-binding and severing protein cofilin. This led to enhanced focal adhesion formation and increased stress fibers. Using single cell force traction measurements, the net effect of HMGB1 signaling was found to be a TLR4-dependent increase in cell force adhesion to the underlying matrix, accounting for the impairment in enterocyte migration. These findings demonstrate a novel pathway by which TLR4 activation by HMGB1 delays mucosal repair, support a role for HMGB1 in the pathogenesis of NEC, and suggest a novel potential therapeutic target in the amelioration of this devastating illness.

1939/B318
The Novel Plasminogen Receptor, Plg-RKT, Facilitates Macrophage Migration and Recruitment.
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Activation of plasminogen, the zymogen of the broad spectrum serine protease, plasmin, is markedly promoted when plasminogen is bound to cell surfaces. Cell-associated plasmin
facilitates a wide array of physiological and pathophysiological processes in which cells must degrade an extracellular matrix in order to migrate, including macrophage recruitment during inflammation. We have recently identified a novel transmembrane plasminogen receptor, Plg-R_{KT}, which is induced when monocytes differentiate. Plg-R_{KT} exposes a C-terminal lysine on the cell surface and promotes plasminogen activation. Therefore, we investigated the role of Plg-R_{KT} in macrophage migration using an anti-Plg-R_{KT} mAb that recognized the C-terminus of Plg-R_{KT} and competed with plasminogen for binding to cells. Chemotaxis assays were performed in transwells using M-CSF-differentiated murine monocyte progenitor cells, expressing high levels of Plg-R_{KT}, as assessed in western blotting. The cells were pre-treated with either anti-Plg-R_{KT} mAb or isotype control and cell migration was stimulated by monocyte chemoattractant protein-1 (MCP-1) in the presence or absence of plasminogen. Plasminogen-dependent cell migration was substantially decreased by 45% in the presence of anti-Plg-R_{KT} mAb compared to the isotype control. Anti-Plg-R_{KT} mAb did not significantly affect cell migration in the absence of plasminogen. To assess cell migration in vivo, 9-week-old C57BL/6 female mice were injected intravenously with either anti-Plg-R_{KT} mAb or control IgG, 30 minutes prior to intraperitoneal injections with 3% thioglycollate, followed by a second injection of antibody 24 hours after thioglycollate treatment. Macrophages were harvested by peritoneal lavage at 72 hours. Anti-Plg-R_{KT} mAb markedly inhibited macrophage recruitment in a dose-dependent manner. At a 500μg dose of anti-Plg-R_{KT} mAb, 5.3×10^5 ± 1.3×10^4 cells were recovered in the peritoneal exudate compared to 1.3×10^6 ± 1.1×10^4 cells in mice injected with control IgG (P<0.001, n=5). These results suggest a major role for Plg-R_{KT} in plasminogen-dependent macrophage recruitment and in regulation of the inflammatory response.

1940/B319
JNK Phosphorylation of a Novel Substrate Stabilizes Actin and Inhibits Cell Migration.
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Cell migration is a fundamental biological event that is critical for developmental and regenerative processes. Deregulated migration can result in extreme conditions such as metastatic cancer and neurological birth defects. Here we demonstrate that an actin regulatory protein P2, is a novel effector of JNK that directs cell movement. JNK phosphorylates three sites on P2, enabling it to bundle/stabilize F-actin and retard cell migration. Conversely, when P2 cannot be phosphorylated on JNK sites, there is an increase in actin mobility and cell migration is enhanced. P2 is predominantly a neuronal protein, and exogenous expression of P2 in non-neuronal cells alters cell migration from a mode that is enhanced by JNK activity to a mode that is retarded by JNK. These data describe a new mechanism whereby JNK regulates cell migration and suggest that the consequence of JNK activation on cell movement, whether facilitating or inhibitory, is dependent on the nature of JNK targets available.

1941/B320 ABSTRACT WITHDRAWN

1942/B321
The Lectin-like Domain of Thrombomodulin Inhibits Melanoma Cell Invasion through Regulation of Matrix Metalloproteinase.
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Thrombomodulin (TM) is a type-I transmembrane glycoprotein with an NH2-terminal C-type lectin-like domain, 6 tandem repeated epidermal growth factor-like structures, an O-glycosylation site rich region, a transmembrane domain and a short cytoplasmic tail. The well-known function of TM is a cofactor for thrombin-dependent activation of protein C in endothelial cells. The wide
expression of TM was discovered in many cell types, including epithelial cells and some tumor cells. The expression of TM on tumor cells is negatively correlated with tumor malignancy. However, the function of TM in these cells is still unclear. In this study, recombinant TM domain proteins were prepared and the biological function of these proteins was investigated. Recombinant TM domain proteins containing lectin-like structure showed inhibitory effect on the invasion of B16F10 melanoma cells in vitro. Recombinant lectin-like domain of TM (rTMD1) effectively inhibited B16F10 melanoma cells invasion and trans-endothelial cell migration in a dose-dependent manner. The expression of matrix metalloproteinase 2 was suppressed by rTMD1 that may through interfering with mitogen-activated protein kinase pathway (MAPK/ERK) induced by fibroblast-cultured media and stromal cell-derived factor-1. Flow cytometry assay revealed that rhodamine-labeled rTMD1 bound to cell surface of B16F10 cells in a dose-dependent manner and the binding of rhodamine-labeled rTMD1 was competed by excess rTMD1, monoclonal antibody directed to the lectin-like domain of TM, recombinant stromal cell-derived factor-1, and polyclonal antibody directed to CXC chemokine receptor 4. The inhibition of the expression of matrix metalloproteinase 2 by rTMD1 was also examined in A549 cells and MDA-MB231 cells. In conclusion, rTMD1 showed inhibitory effect on tumor cell invasion which may be through down-regulation of matrix metalloproteinase 2.

Prokaryotic Cytoskeletal Systems (1943 – 1949)

1943/B322
Structure and Dynamics of the MreB Bacterial Cytoskeleton.
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Shape maintenance in rod-like bacteria is regulated by the actin homolog MreB. To determine the localization pattern of MreB at normal expression levels under endogenous regulation, we used mCherry-tagged MreB replacing the chromosomal gene under its native promotor. We recorded epi-fluorescent images in live and fixed E. coli cells. Our results suggest that the normal MreB cytoskeleton does not have a continuous helical structure, as previously suggested from observations on overexpressed YFP-MreB. Using statistical and image analysis tools we measured the spatial parameters of MreB cytoskeleton in hundreds of bacterial cells. Our data suggest that MreB is arranged in two sections, each combining helical and ring-like segments, separated at the future division site. Furthermore, while MreB dynamics were previous reported to be slow (and comparable to the bacterial doubling time), our time-lapse microscopy shows that MreB structures at normal expression levels undergo rapid reorganization within few minutes. We have examined the effects of various pharmacological perturbations on the MreB localization and dynamics. Our results call into question the current view of MreB organization and its role as a structural element in the bacterial cell.

1944/B323
Distinct Morphological Classes Obtained with Non-Lethal Mutations in mreB in the Bacterium.
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The mechanism by which bacteria coordinate the spatial pattern of synthesis and degradation of their peptidoglycan cell wall to maintain their cell shape during growth and division is not well understood. This process is known to require the gene mreB, which encodes a member of the actin superfamily, in the rod-like bacteria E.coli, B.subtilis, and C.crescentus. To investigate the role of MreB in cell shape maintenance, we used the drug A22 to select for spontaneous mutations in mreB in Caulobacter crescentus. We obtained several mutant strains, all of which contain single lesions in the mreB gene and repeatedly grow in the presence or absence of A22. Upon observing the cell shapes in each strain, we identified three morphological mutant classes.
Class a mutants are indistinguishable from wild type in length, width, and curvature. Class B mutants are longer, thinner, and frequently sigmoidal. Class C mutants are wider and pleiomorphic. A subset of class C mutants was found to have a dramatically altered morphology, with an enlarged, roughly spherical cell body and long, thin extensions of the cell poles. GFP-MreB was found to localize to the thin polar extensions and to be excluded from the enlarged cell bodies, and timelapse microscopy showed that this strain actively elongates these extensions, often generating new extensions from the new pole immediately after division. Mapping of the mutant residues onto the known crystal structure for *T. maritima* MreB1 showed that the residues in each class are clustered together in specific regions of the structure, suggesting that regions of the protein structure can be linked to distinct morphological phenotypes. We hope that these mutant strains will provide a new tool with which to connect MreB’s protein structure to its function in bacterial cell shape.

1945/B324

**The Structure and Assembly Dynamics of Plasmid Actin AlfA Imply a Novel Mechanism of DNA Segregation.**

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Bacterial cytoskeletal proteins participate in a variety of processes, including cell division and DNA segregation. Polymerization of one plasmid-encoded, actin-like protein (ALP), ParM, segregates DNA by pushing two plasmids in opposite directions and forms the current paradigm for understanding active plasmid segregation. An essential feature of ParM assembly is its dynamically instability, the stochastic switching between growth and disassembly. It is unclear whether dynamic instability is an essential feature of all ALP-based segregation mechanisms or whether bacterial filaments can segregate plasmids by different mechanisms. We expressed and purified AlfA, a plasmid-segregating ALP from *Bacillus subtilis*, and find that it forms filaments with a unique structure and biochemistry: AlfA nucleates rapidly; polymerizes in ATP or GTP; and forms highly twisted, ribbon-like, helical filaments with a left-handed pitch and protomer nucleotide binding pockets rotated away from the filament axis. Intriguingly, AlfA filaments spontaneously associate into uniform-sized, mixed-polarity bundles. Most surprisingly, our biochemical characterization reveals that AlfA does not display dynamic instability and is relatively stable in diphosphate nucleotides. These results: (i) reveal a remarkable structural diversity among bacterial actin filaments and (ii) indicate that AlfA filaments partition DNA by a novel mechanism.

1946/B325

**Chromosomal Loci Move Subdiffusively Through a Viscoelastic Cytoplasm.**

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Particle-tracking of fluorescently labeled chromosomal loci in live *E. coli* cells reveals a robust scaling law, with mean square displacement (MSD) ~ t^0.4. We use Brownian dynamics simulations to show that this anomalous behavior cannot be fully accounted for by the Rouse or reptation models for polymer dynamics. Instead, the motion seems to arise from the interaction of the DNA polymer with the viscoelastic environment of the cytoplasm. The time-averaged and ensemble-averaged MSD exhibit ergodicity and the autocorrelation of displacements in individual trajectories is negative. Our results are most consistent with fractional Brownian motion and rule out a continuous time random walk model. Finally, we present a general analytical derivation for the scaling of a monomer in a polymer within a viscoelastic medium.

1947/B326

**Mycobacterial FtsZ Proteins as Target for Benzimidazole Compounds: Structural View.**

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FtsZ proteins responsible for division of prokaryotic cell exhibit a high amino acid sequence conservativity as well as spatial organization and, thus, are exceptionally attractive targets for new antibacterial (in particular, antituberculosis) drugs. Benzimidazole derivates blocking bacterial cell division through interaction with FtsZ proteins seem to be very perspective candidates for rational drug design. Therefore understanding of structural mechanism of mycobacterial FtsZ-benzimidazole interaction with next identification of appropriate binding site(s) is key moment in development new drugs against tuberculosis. Spatial structure of FtsZ from Mycobacterium tuberculosis (PDB entry 1RLU) was optimized using AMBER force field parameters and Polak-Ribiere algorithm (conjugate gradient). Obtained structure were undergone to 3D-alignment with animal (Haemonchus contortus and Homo sapiens) and fungal (Neurospora crassa and Mycosphaerella graminicola) β-tubulins and their complexes with carbendazim. Amino acid sequences of corresponded proteins also were aligned and compared among themselves. Accordingly to spatial structure alignment, the β-strands S4 (95-99 residue), S5 (122-130 residues), S6 (155-161) and S8 (256-262) and α-helixes H1 (18-29) and H6 (106-109) exhibit the most spatial structure similarity to appropriate regions of β-tubulin molecules. Among them S5, S6, S8 and partly S4 strands clearly correspond to β-tubulin regions directly involved in interaction with benzimidazoles. With high probability it can suppose that benzimidazole bind with the same areas of FtsZ and β-tubulins. Possible binding site in Mycobacterium case contains residues Phe97, Leu124, Thr125, Val126, Gly127, Val128, Cys155, Asp156, Thr157, Leu158, Ile159, Ile161, Leu187, Gly190, Val191, Ile194, Me215. Amino acids in positions 97, 128, 156, 161 are identical with appropriate β-tubulin residues, residues in positions 125, 126, 127, 159, 187, 191 194, 215 are conservative. This work is supported by cooperative STCU-NASU grant 4932 "In silico design and synthesis of compounds with antituberculous activity on the base of their structural interaction with FtsZs".

1948/B327
Roles of the Direct MinE-Membrane Interaction in Proper Function of the Min System.
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Proper placement of the cell division site in E. coli requires coordinated action of three proteins: MinC, MinD and MinE. It was previously known that MinE functions in the system through stimulating the MinD ATPase activity. In this study, we report evidence of direct MinE-membrane interaction that contributes to proper function of the Min system. Using the In Vitro sedimentation assays, we detected interaction between MinE and liposomes reconstituted from E. coli phospholipids. The degree of sedimentation was strongly enhanced by more than five folds when the N-terminal domain of MinE was used alone. We thought that electrostatic force contributes to the interaction based on the results that MinE sedimentation with liposomes was prevented by increasing salt concentration and by the addition of cationic phospholipids, but enhanced by the presence of anionic phosphatidylglycerol and cardiolipin. Meanwhile, a mutant MinE1-31-Yfp construct carrying substitutions in the basic residues R10, K11, and K12 showed dispersed fluorescence in cells that was in contrast to the peripheral localization of the wild-type construct. Furthermore, the same mutant appeared to be defective in formation of the MinE ring, thus unable to support normal oscillation cycles of MinD. on the other hand, the purified mutant protein was able to stimulate MinD ATPase activity effectively similar to the wild-type protein. In summary, direct MinE-membrane interaction appears to be necessary for supporting formation of the MinE ring and proper function of the Min system.

1949/B328
Identification of Regulatory Networks that Integrate Protein Localization and Gene Expression to Control Bacterial Cell Cycle Progression.
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Regulatory networks consisting of many inter-connected elements ensure reliable cell cycle progression and developmental transitions in bacterial cells. Notably, these networks rely on the dynamic three-dimensional deployment of signaling molecules and transcription factors. We wish to understand the nodes, the topology, and the interactions of these networks in the bacterial model system Caulobacter crescentus. Each Caulobacter cell division produces two morphologically and functionally distinct progeny: a swarmer cell and a replication-competent stalked cell. The swarmer cell then differentiates into a stalked cell after shedding its flagellum and growing a stalk capped by a holdfast. Key to the regulation and timing of this developmental transition are two histidine kinases, PleC and DivJ, which localize to opposite ends of the cell at different stages of the cell cycle. These proteins, along with the CpaE structural protein, were fluorescently labeled using chromosomal fusions to create a multiply labeled reporter strain. A mutant library was created using a conditional Tn5 transposon derivative with a synthetic linker specifically designed for high volume mapping of transposon locations via a semi arbitrary PCR. The mutant library was passed through a pipelined automated live cell microscopy procedure that included image acquisition, image processing, pattern recognition and statistical learning algorithms. We identified four statistically significant and systematically robust clusters of gene disruptions causing changes in the cell cycle timing and sub-cellular localization of reporter molecules. The clusters map into distinct regulatory pathways, and one of the clusters comprises six genes directly implicated in control of the swarmer to stalked cell transition.

Invertebrate and Mammalian Development (1950 – 1967)

1950/B329
Secretion of Hedgehog in Different Complexes.
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The signaling protein Hedgehog (Hh) carries two lipid modifications that confer a high affinity to cellular membranes, but is nevertheless secreted in soluble complexes that move through the extracellular space. Long-range Hh signaling in Drosophila imaginal tissues requires association with the lipoprotein Lipophorin (Lpp), whose scaffolding protein is homologous to human ApoB. Short-range signaling does not require Lpp, suggesting that long and short-range Hh signaling might depend on different forms of secreted Hh. We seek to determine the molecular composition of the different secreted Hh complexes, the cellular requirements for their formation, and the structural basis of their assembly. We make use of the previously not recognized pool of Hh that is released into the hemolymph, the body fluid of Drosophila; this system makes the In Vivo secretion of Hh accessible to biochemical analysis and uncouples it from downstream processes like uptake into receiving cells and signaling. Secreted Hh quantitatively cofractionates with Lpp in wild type animals. When Lpp levels are limiting, either due to Lpp knockdown or Hh over-expression, Hh is released in distinct complexes of higher density. We considered whether a second ApoB-like lipoprotein that is encoded by the Drosophila genome might contribute to the formation of Lpp-free Hh complexes, but found that Hh does not associate with it. Whether Lpp-free Hh complexes contain other proteins or lipids remains to be established. To distinguish structural features of Hh required for incorporation in Lpp particles or Lpp-free complexes, we utilized a series of Hh mutants. We show that Hh lipid modifications are required for Lpp association, while not being required for Hh secretion. In contrast, Hh homodimerization and heparan sulfate binding are dispensable both for secretion and for the formation of Hh-lipoprotein complexes. Taken together, these data indicate that Hh can be released in both Lpp-associated and Lpp-free forms, with signaling properties that may differ.

1951/B330
The Role of Canoe/Afadin in Regulating the Linkage between Actin and Adherens Junctions during Morphogenesis.
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Adherens junctions (AJs) mediate cell adhesion and couple adhesion and cell shape change by linking the actin cytoskeletons of adjacent cells. This linkage was thought to be direct, via the catenins, but recent work cast this into doubt. The nectin-afadin complex also localizes to AJs and links to the actin cytoskeleton. Mammalian afadin has been suggested to be essential for adhesion and polarity establishment, but its mechanism of action is unclear. In contrast, Drosophila’s afadin homolog Canoe (Cno) has suggested roles in signal transduction during morphogenesis. We completely removed Cno from embryos, testing these hypotheses. Surprisingly, Cno is not essential for initial AJ assembly, or for AJ maintenance in many tissues, suggesting that at least in Drosophila the nectin-afadin system is not essential for cell-cell adhesion. However, morphogenesis is impaired from the start. Apical constriction of mesodermal cells initiates but is not completed. The actomyosin cytoskeleton disconnects from AJs, uncoupling actomyosin constriction and cell shape change. Cno is strongly enriched in tricellular junctions, where it co-localizes with a subset of actin, suggesting a key role for these structures in apical constriction. Cno has multiple direct interactions with AJ proteins, but is not a core part of the cadherin-catenin complex. Additionally, Cno does not require either the cadherin-catenin complex or the nectin Echinoid for its cortical localization. Instead, Cno localizes to AJs by a Rap1 and actin-dependent mechanism. These data suggest Cno regulates linkage between AJs and the actin cytoskeleton during morphogenesis; we suspect different proteins may serve this role in different places, as Cno is only required for a subset of the events in embryogenesis that should require AJ-actin connections. We are currently exploring what partners work with Cno and Rap1 in apical constriction. While Cno and Rap1 are not required for AJ maintenance in most tissues, they do play a critical role in the ventral ectoderm. We are examining the role of Cno and Rap1 in AJ maintenance in this dynamic tissue and determining whether this reflects effects on adhesion, cell polarity or cytoskeletal contractility.

1952/B331
Forces from the Amnioserosa are Sufficient for Native or Nearly Native Rates of Dorsal Closure in Drosophila Embryos.
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The morphogenetic movement of dorsal closure in Drosophila embryogenesis occurs via four coordinated biomechanical processes. Remarkably, embryos continue closure at native rates after complete removal of two of the processes. The following reveals the significance of this finding. At the beginning of closure, the dorsal side of the embryo consists of a roughly eye-shaped opening filled with a tissue called the amnioserosa and flanked by the lateral epidermis, at the leading edge of which, is an actomyosin-rich cable or purse string. During closure, the eye-shaped structure closes. Biophysical investigation of closure through mechanical jump experiments involves steering a laser microbeam to cut tissue and time-lapsed imaging to record the response. Such experiments indicate that the amnioserosa and the purse string both contribute forces for closure. In contrast, the lateral epidermis produces a force that opposes closure, presumably due to dorsal-ward stretching. The canthi (the corners of the eye) anchor the leading edge of the lateral epidermis to provide curvature which resolves tension in the purse strings into dorsal-ward forces that contribute to closure. At the canthi, the zipping process finalizes the association of opposing lateral epidermal sheets to form a continuous epithelium and serves to maintain purse string curvature throughout closure. Individual tissue forces are significantly larger than the net applied force. To investigate the collective contribution of the contractile purse strings and the process of zipping, we used laser cuts to sever the canthi from their connections to the amnioserosa and the purse strings. After recoil, closure resumes and purse strings flatten into two parallel fronts. With no purse string curvature (no closure-promoting purse string force) and no zipping, closure continues at native or nearly native rates to completion. Thus, the amnioserosa is sufficient to drive the complex morphogenetic movement of dorsal closure. We hypothesize that amnioserosa cells up-regulate their closure-promoting force to compensate for the loss of contributions from the purse strings and zipping. The mechanism for this remains an open question. Grant Support: GM33830.
Ascidians are close relatives to vertebrates, and form a neural tube of only a few hundred cells, providing an unparalleled opportunity to study this fundamental morphogenetic process at the cellular level. Several distinct subprocesses contribute to ascidian neurulation. First, primary invagination of the neural plate is followed by axial elongation accompanied by mediolateral cell-cell intercalation and oriented cell divisions. Second and concurrently, constriction of the blastopore and medially-directed crawling bring epidermal cells together at the posterior midline to initiate neural tube closure. Finally, neural tube closure proceeds through anterior to posterior zippering of both the neural tube and the overlying epidermal cells. Experiments with explants and laser ablation reveal that neural plate invagination proceeds normally in the absence of the posterior zipper-forming cells, but zippering fails. Conversely, zipper initiation and propagation proceed normally in the absence of the primary neural plate. Furthermore, unlike in vertebrates, formation and elongation of the neural tube does not require the presence of a notochord. Time-lapse imaging and immunostaining of fixed embryos show that a combination of local protrusive extension and contraction drive the initiation and propagation of the zipper. A propagating zone of localized rho-kinase-dependent myosin activation accompanies and is required for blastopore closure and for the initiation and progression of zippering, but not for primary invagination of the neural plate. Thus in ascidians, the complex process of neurulation can be decomposed into, and understood in terms of, a set of fundamental cellular mechanisms that are directly amenable to experimental analysis.

Ascidians are basal Chordates that share with Vertebrates a similar body plan, including a dorsal nerve chord. Small cell numbers, optical transparency and experimental accessibility make them superb model organisms for studying the molecular basis, cytomechanics, and regulation of nerve chord morphogenesis. The ascidian neural tube forms by neurectoderm folding, followed by fusion of the neural folds at the dorsal midline by a posterior-to-anterior "zippering" process. Using time-lapse fluorescence microscopy, we found that neural closure is accompanied by the constriction of a supra-cellular purse string-like actin cable that develops at the boundaries between presumptive neurectoderm and lateral epidermis. Significantly, constriction of the purse string is strongly biased to it’s posterior end where it is accompanied by highly localized protrusive activity and cortical contractility. Immuno-staining of fixed embryos and time-lapse microscopy of live embryos revealed that the active RhOa and active (phosphorylated) forms of Myosin are similarly localized to the highly contractile posterior zone, which propagates posterior to anterior and cell to cell as zippering proceeds. Blocking the RhO-pathway kinase ROCK with the inhibitor Y-27632 leads to loss of localized activated Myosin and specifically causes the zipper to collapse and the entire purse string to relax. Following fusion of opposed neural fold cells, midline cells are first dragged anteriorly as the zipper progresses further forward, then abruptly released as the apical domains of presumptive dermal and neural cells lose contact. The result of this process is to form two epithelia (one neural, one dermal) from a single initial
epithelial layer. Our data highlight the existence of a “morphogenetic organizer” that coordinates supercellular force generation in time and space to achieve the completion of an essential step in chordate development.

1955/B334
Projections on Goniosoma spelaeum Spermatozoa: Where Do They Come from?
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The morphological diversity found in spermatozoa of the Arachnida orders allows its division into two big groups: one presenting flagellate spermatozoon and other presenting aflagellate spermatozoon. The first group develops elongate flagellate spermatids that roll up in the final process of spermiogenesis to form spherical structures with retracted axonemes. The second group presents a wide morphological variety, probably determined by the shape of the nucleus since almost all cytoplasm is eliminated during the spermatozoa development. This group comprises the order Opiliones, which includes a great variety of forms such as spherical, filiform and flattened. Despite this, studies on harvestman spermatozoa are scarce and old, and focused mainly on species from the North Hemisphere. Recent works about morphology and ultrastructure of spermatozoa in Laniatores, a suborder of Opiliones, reported the presence of projections on its surface, which function could be related, for example, to the sperm transport through the male reproductive tract or the anchorage onto the female tract. This hypothesis proposes that these projections would be extracellular structures because they were only observed in mature spermatozoa from the seminal vesicle. The present study focused on the spermatozoa of Goniosoma spelaeum, another laniatorean species that present these projections. Different techniques of microscopy were applied in order to verify how these structures are formed. The images from transmission and scanning electron microscopy indicated that projections are individual structures composed only by cytoplasm surrounded by cytoplasmic membrane. Confocal microscopy showed a net of actin and tubulin laying under the membrane. These results offer support for our hypothesis that the projections are formed during the spermatogenesis. During the development of the spermatozoon the net of actin and tubulin compresses the cytoplasm which leaks through the meshes of the net, originating the projections found in mature cells.

1956/B335
Inorganic Polyphosphate Inhibits an Aspartic Protease-Like Activity in the Eggs of Rhodnius prolixus (Stahl) and Impairs Yolk Mobilization In Vitro.
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Inorganic polyphosphate (poly P) is a linear polymer of phosphate (Pi) residues that has been shown to act as modulator of some vertebrate cathepsins. In the egg yolk granules of Rhodnius prolixus, a cathepsin D is the main protease involved in yolk mobilization and is dependent on an activation step mediated by acid phosphatases. In this study, we provide evidence for a role of poly P stored inside yolk granules on the inhibition of cathepsin D and arrest of yolk mobilization during early embryogenesis of these insects. Enzymatic assays detected poly P stores inside the eggs of R. prolixus. We observed that micromolar poly P concentrations were able to inhibit cathepsin D proteolytic activity using both synthetic peptides and homogenates of egg yolk as substrates. Poly P was also a competitive inhibitor of an acid phosphatase activity. Fusion events have been suggested as important steps towards acid phosphatase transport to yolk granules. We observed that poly P levels in those compartments were reduced after In Vitro fusion assays and that the remaining poly P did not have the same cathepsin D inhibition activity after fusion. Our results are consistent with the hypothesis that poly P is a cathepsin D inhibitor and a substrate for acid phosphatase inside yolk granules. It is possible that, once activated, acid phosphatase might degrade poly P, allowing cathepsin D to initiate yolk proteolysis. We, therefore, suggest that degradation of poly P might represent a new step toward yolk mobilization during early embryogenesis of R. prolixus.
The nematode eggshell is a rigid, impermeable structure that protects the embryo during development, and is required for the first embryonic cell division. The essential function of the eggshell makes it an attractive drug target to combat parasitic nematode infection, but little is known about eggshell composition or the process by which it forms. We have identified the first structural proteins required for eggshell formation in the nematode *C. elegans*. CPG-1 and CPG-2 are functionally redundant chondroitin proteoglycans that also contain chitin-binding domains, consistent with a role in assembly of the chitinous eggshell. Live imaging of fluorescent strains shows CPG-1 and CPG-2 are secreted from caveolin-enriched cortical granules soon after fertilization, while immuno-electron and fluorescence microscopy show they associate with the inner eggshell layer and are distributed throughout the perivitelline space. To better understand the function of chondroitin proteoglycans in eggshell assembly, we depleted CPG-1, CPG-2, and SQV-5 (the chondroitin synthase) by RNAi. In utero, embryos co-depleted of CPG-1/CPG-2 or chondroitin have identical phenotypes, exhibiting defects in eggshell osmotic integrity and early embryonic cell divisions. However, when osmotic support is provided to dissected embryos, early embryonic divisions are robustly rescued in SQV-5 depletions, but only partially rescued in CPG-1/CPG-2 co-depletions. Ultra-structural analysis shows embryos depleted of chondroitin form all three eggshell layers, while embryos co-depleted of CPG-1/CPG-2 fail to form the third inner layer. Correlation of structural and functional data suggests CPG protein cores may have a function separable from that of chondroitin chains during eggshell assembly, and provides evidence for the first structural proteins required for eggshell formation.

Embryonic development depends on the provision of morphogenetic instructions generated by signaling factors such as those belonging to the transforming growth factor-beta superfamily that cells receive in an autocrine or paracrine fashion. These instructions contribute towards the determination of cell lineage and differentiation, tissue growth and tissue patterning in the primary body axes. Nodal is a member of the TGF-β superfamily, the signaling pathway transduced by which plays crucial roles during early vertebrate development. Type IIA procollagen (IIA) is an alternatively spliced isoform of type II procollagen and is an extracellular matrix protein that binds to BMP-2, 4, 7 and TGF-β1 in vitro. It is known that modulation of activity of signaling molecules can be mediated by ECM and it has been proposed that IIA may regulate early embryonic patterning by either facilitation or inhibition via binding to components of these pathways. However, the essential role of IIA in early embryogenesis has not been established. Here we show that IIA facilitates Nodal signaling in vitro. IIA synergizes with FoxH1 to facilitate Nodal signaling in the absence of exogenous Cripto. We also find that IIA binds to both pro-Nodal and mature Nodal ligand, and strengthen the interaction between Nodal and Cripto. Together with the observation that IIA interacts with the Nodal type I receptor ALK-4, we hypothesize that IIA facilitates formation of the active signaling complex which contains Nodal, Cripto and ALK-4 proteins. In the mouse embryo, expression of Nodal target genes such as *Pitx2* and *Nodal* were either altered or dramatically down-regulated in the node and the left lateral plate mesoderm (LPM) in a null mutant of procollagen IIA at E8.5. Our results demonstrate IIA positively regulates
Nodal signaling. Result from this work will lead to a better understanding of the unrevealed roles of ECM proteins in early vertebrate embryogenesis.

1959/B338
Arginylation Regulates Neural Crest Cell Migration and Craniofacial Morphogenesis.
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Protein arginylation is a poorly understood posttranslational modification that is essential for mouse development. Knockout of arginyltransferase (Ate1) in mice leads to embryonic lethality with global morphogenic defects that are reminiscent of cell migration-dependent phenotypes seen in other mouse models. Recent studies implicate protein arginylation in cell migration and the functioning of actin cytoskeleton in culture, leading to a hypothesis that Ate1 function regulates cell migration during morphogenesis. To test this hypothesis, we produced Wnt1-Cre Ate1 conditional knockout mice (Wnt1-Ate1), with Ate1 deletion driven by Wnt1 promoter in neural crest cells, which migrate over long distances to give rise to organs and tissues throughout the body. Wnt1-Ate1 mice die at birth and in the first 2-3 weeks after birth with severe breathing problems and growth and behavioral retardation, indicating the critical role of Ate1 in the functioning of the neural crest cells. Wnt1-Ate1 pups have prominent defects in tissues derived from cranial neural crest cells, including short palate and altered opening to the nasopharynx, which likely contribute to the abnormal breathing and early death. In addition, Wnt1-Ate1 mice have cranial malformations and facial defects, originating in the abnormalities of the frontal bones. No defects are seen in other neural crest-derived structures of Wnt1-Ate1 mice. Reporter studies using mice with LacZ-labeled neural crest cells show that neural crest cell migration patterns in Wnt1-Ate1 fetuses are different from their wild type littermates, and Ate1 knockout cells in culture show an overall delay in motility, likely regulated by intracellular mechanisms rather than extracellular signaling events. Taken together, our data suggest that arginylation plays a general role in the migration of the neural crest cells in development, by regulating the molecular machinery that underlies cell migration through tissues and organs during morphogenesis.

1960/B339
βcyto-Actin but not γcyto-Actin Is Essential for Embryonic Viability.
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The complex process of development undoubtedly requires a properly regulated actin cytoskeleton, however, the contributions of different actin isoforms during embryogenesis remains to be investigated. Of the six closely-related actin isoforms, each encoded by distinct genes, the ubiquitously expressed βcyto- and γcyto-actins differ at only 4 out of 375 amino acids. We have used the same gene targeting strategy to generate null alleles of the Actb and Actg1 genes and our characterization of the resulting isofrom-specific null animals demonstrates that βcyto-actin but not γcyto-actin is essential for embryonic viability. γcyto-Actin null mice were found at the expected Mendelian ratio from embryonic day 9.5 through 18.5, but exhibited stunted growth during embryonic and postnatal development as well as delayed cardiac outflow tract formation that resolved by birth. Interestingly, less than 50% of γcyto-actin null mice survived 24 hours after birth and neonatal lethality appeared to be due to respiratory failure. In sharp contrast, βcyto-actin null mice were fully embryonic lethal by day 7.5 of gestation. Furthermore, mice with only one functional copy of the βcyto-actin gene were live born at the expected Mendelian ratio, but exhibited a reduced survival rate beginning at approximately four weeks of age. Together, these data indicate that βcyto-actin is essential for viability while γcyto-actin is important but not required.
for survival. The strikingly different phenotypes of βcyto- and γcyto-actin null mice suggest distinct functions for βcyto- and γcyto-actin during embryonic development.

1961/B340
Identification of Neuronal Nuclei (NeuN) as Fox-3 which Plays a Role in the Alternative Splicing of Non-muscle Myosin Heavy Chain II-B.
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Fox-3 (also called hexaribonucleotide binding protein 3 and D11Bw90517e) contains an RNA recognition motif and is classified as a member of the Fox-1 gene family which binds specifically to the RNA element, UGCAUG. We demonstrate that Fox-3 functions as a splicing regulator using a model minigene system of non-muscle myosin heavy chain II-B. Experiments with shRNA and complement rescue experiments using P19 cells show that Fox-3 induced during neural differentiation can activate inclusion of a neural cell-specific alternative exon. Recently, in characterizing the properties of Fox-3 we have noted the similarity between its cellular localization and that of NeuN (Neuronal Nuclei), a neuron-specific nuclear protein which is identified by immuno-reactivity with the monoclonal antibody, anti-NeuN. Anti-NeuN has been used widely as a reliable tool to detect most post-mitotic neuronal cell types. To date, however, the identity of its antigen, NeuN itself, has been unknown. Here, we identify NeuN as the Fox-3 gene product by providing the following evidence: 1) Mass spectrometry analysis of anti-NeuN immuno-reactive protein yields the Fox-3 amino acid sequence. 2) Recombinant Fox-3 is recognized by anti-NeuN. 3) shRNAs targeting Fox-3 mRNA down-regulate NeuN expression. 4) Fox-3 expression is restricted to neural tissues. 5) Anti-Fox-3 immuno-staining and anti-NeuN immuno-staining overlap completely in neuronal nuclei. We also show that the synaptic vesicle protein, synapsin I is cross-reactive with anti-NeuN. However, anti-NeuN recognizes synapsin I in immunoblots with one-order of magnitude lower affinity than Fox-3 and does not recognize synapsin I using immuno-histology. Identification of NeuN as the splicing factor, Fox-3, clarifies an important element of neurobiology research.

1962/B341
Role of Epigenetics (Dnmt-1-mediated DNA methylation) in Retinal Differentiation.
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Objective: DNA methylation is considered important for multipotential progenitor cell to acquire the restricted cell fate choice and is mediated by three known DNA methyltransferases: Dnmt1, Dnmt3a and Dnmt3b. Two of them (Dnmt3a and Dnmt3b) are known to be responsible for de novo methylation of DNA, while Dnmt1 is considered the maintenance methyltransferase and replicates the DNA methylation pattern on the daughter DNA strand in dividing cells. DNA methylation is generally associated with downregulation of gene expression. A well-studied developmental effect of DNA methylation is stable silencing of gene expression. Literature evidence suggests that site-specific DNA methylation may help to stabilize the postmitotic cell fate choice by setting the expression pattern in a given cell needed for its function. We provide some initial evidence that retinal cell fate specification and differentiation may be subject of such epigenetic mechanisms. Methods: We are generating a series of conditional retina-specific knockouts of the three DNA methyltransferases using Rx-Cre mouse driver line. Histological data in retinas of Dnmt1 conditional knockout mice were done by light and electron microscopy using development stages from e16.5 to post-natal day16.5. Confocal immunofluorescence studies were done on retinal sections using a series of retina-specific antibodies to photoreceptors, RPE and Muller cells, and second order neurons to investigate the key changes influenced by retina-specific ablation of Dnmt1. Results: Severe morphological changes of outer nuclear layer were
observed in the retinas of Dnmt1 knockout animals. The pathology included severely shortened or absent photoreceptor outer segments, retinal folding and pseudorosette formation, regions of attenuated RPE and abnormalities in retinal lamination, thinning of ONL, and reduction of cone numbers. Conclusions: Our studies emphasize the first evidence of the importance of Dnmt1-mediated epigenetic mechanisms guiding mouse retinal development.

1963/B342
Fat3 Cadherin Regulates Dendrite Morphology and Cell Migration in the Vertebrate Retina.
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During the assembly of neural circuits, newly born neurons migrate to specific locations and extend processes in stereotyped manners to contact the appropriate synaptic partners. We find that the large atypical cadherin Fat3 coordinates these events during development of the vertebrate retina. Following terminal mitosis most retinal amacrine cells migrate to the inner nuclear layer (INL) and a smaller set of displaced amacrine cells migrate to the ganglion cell layer (GCL). Cells from both classes extend polarized arbors of dendrites into the inner plexiform layer (IPL). We show that fat3 mRNA is expressed by amacrine cells and ganglion cells and that Fat3 protein is present throughout the developing IPL. In retinas from fat3 knockout mice there is increased amacrine cell migration into the GCL leading to thickening and disorganization of this layer. In addition fat3 mutant amacrine cells develop atypical bipolar dendritic arbors and extend additional dendrites away from the IPL. As a result two novel synaptic plexiform layers are formed in the knockout retina. The first is located within the INL and contains ectopic dendrites extending from amacrine cells remaining in the INL. The second is interspersed with the ganglion cell axons and contains ectopic dendrites extending from displaced amacrine cells. Formation of the indigenous plexiform layers is unaltered thus the fat3 knockout retina contains five plexiform layers. We further demonstrate that these developmental events are regulated by two Fat3 signaling mechanisms that function in parallel: A cell-autonomous mechanism in the amacrine cells that directs dendritic morphology and a non-autonomous mechanism in ganglion cells that regulates amacrine cell migration. Therefore the formation of ectopic plexiform layers can be initiated by amacrine cells alone while the organization of the nuclear layers depends on Fat3-mediated signaling between ganglion cells and amacrine cells. These results provide the first evidence that Fat cadherins regulate neuronal morphology and connectivity.

1964/B343
SUMOylation Modulates the Transcriptional Regulatory Function of Rod Differentiation Factor NRL.
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Purpose: NRL (Neural Retina Leucine zipper) is the key transcription factor, essential and sufficient, for rod differentiation in mouse retina. NRL is a member of the Maf transcription factor family and its activity is regulated by post-translational modifications. We have demonstrated that multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. This study was undertaken to investigate the role of SUMOylation in regulating the biological activity of NRL.

Methods: SUMOplot analysis was used to predict potential SUMOylation sites within the NRL protein sequence. SUMOylation of NRL was investigated in an In Vitro SUMOylation assay using purified NRL-GST protein and by immunoprecipitation from transfected HEK293T cells and mouse retina. Different SUMOylation mutants of NRL were generated by site-directed mutagenesis. Their transcriptional activity was assessed in HEK293 cells using luciferase reporter activity assays. NRL mutants were electroporated into the retina of newborn Nrl⁻/⁻ mice, and differentiation of cones and rods was assessed by immunohistochemistry after 21 days.

Results: SUMOplot analysis predicted two lysine residues at position 20 and 24 of NRL as potential sites for SUMOylation. We demonstrate that NRL is indeed SUMOylated both In Vitro and in vivo. The molecular weight of SUMOylated NRL is around 50 kDa, suggesting that two SUMO-1 proteins are linked to NRL. NRL-K20R, NRL-K24R. NRL-K20R/K24R mutants show
lower transcriptional activity on rhodopsin and NR2E3 promoters when expressed alone or co-
expressed with CRX. Misexpression of the NRL-K20R/K24R mutant in Nrl" mice leads to
photoreceptors expressing both rod and cone specific marker proteins (instead of either rod or
cone as is the case in wild type). The overall production of rhodopsin-positive cells is also
decreased in mutants compared to NRL WT. Finally we demonstrate that Nrl interacts with
PIAS3, an E3-SUMO ligase expressed in rod photoreceptors. Conclusions: Our results show that
SUMOylation is important for modulating NRL activity and for appropriate development of rod
photoreceptors.

1965/B344
Role of Primary Cilia in Mammalian Skin Homeostasis and Development.
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The primary cilium is a non-motile, microtubule-based organelle extending from the surface of
most mammalian cells, including cells in the skin. The mammalian skin is a stratified, squamous
epithelium that is continually renewed from keratinocytes that reside in the basal layer of the
epidermis. These basal cells give rise to the terminally differentiated and stratified layers of the
epidermis that replace the upper layer as it is shed. Recent data indicate that the cilium is an
important regulator of several signaling pathways, including the sonic hedgehog (Shh) and
wingless-int (Wnt) pathways, raising the possibility that this organelle may be important for skin
organogenesis and maintenance. To test this hypothesis, we disrupted ciliogenesis in different
domains of skin using the Cre-LoxP conditional mutagenesis approach. Ciliogenesis was
interrupted in the dermis and in epidermal basal keratinocytes using the Prx1-cre and K14-cre
transgenic lines, respectively. In the dermal cilia mutants, hair follicle morphogenesis was
arrested due to impaired Shh signaling. In contrast, the epidermal mutants develop a "ridged" hair
patterning defect, along with mild progressive alopecia. Histological and molecular marker
analyses have revealed an increase in epidermal stratification, accompanied by ingrowths of the
epidermis into the dermis. This is associated with alterations in the orientation of cell division of
the proliferating cells in the basal layer and with altered expression of differentiation markers.
These results are consistent with an expansion of the interfollicular stem cell population and/or a
partial block in progenitor cell differentiation. Ongoing studies aim to fully characterize the
morphological effects of cilia disruption in these mutants, as well as to analyze the molecular and
cellular underpinnings of this phenotype.

1966/B345
Insulin Sensitivity of Placental Mmp14 Is Developmentally Regulated and Differs Between
Arterial and Venous Endothelial Cells: Implications for Angiogenesis Regulation.
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Objectives: Insulin receptor (IR) location shifts during human placental development (Diabetologia
49:123, 2006) from the trophoblast in the 1st trimester to the endothelium at the end of gestation.
Here we hypothesized also a shift in insulin effects from the 1st trimester T to arterial and venous
placental endothelial cells (EC) at term. Matrix metalloproteinases (MMP) are implicated in
trophoblast invasion, angiogenesis and in proliferation, processes all crucial for placental
development. MMP14, one key MMP, is an insulin-sensitive target gene in 1st trimester T
(Diabetes 57:150, 2008). Hence, MMP14 was used to demonstrate the developmental shift in
insulin sensitivity during gestation. Because arterial and venous EC phenotypes differ
(Differentiation 76:1031, 2008), both were included into the study. Methods: IR protein expression
was measured in primary human 1st and term T, and term arterial and venous EC. All cells were
cultured 48 h in presence or absence of 1nM insulin. MMP14 protein levels were measured by
western blot analysis. Inhibitors of PI3K and ERK1/2 signalling were used to identify signalling
pathways accounting for the potential regulation. Results: 1st trimester T highly express IR
whereas at term T show minor expression. at term, EC and here particularly the arterial EC express IR (1st trimester T>A>V>term T). Insulin sensitivity of MMP14 expression paralleled this pattern. MMP14 was upregulated by insulin in primary 1st trimester T (+113%; p=0.05) but unchanged in term T. In arterial EC insulin stimulated MMP14 (+32%; p=0.04) whereas no stimulation was found in venous EC. In both insulin-sensitive cell types, the insulin effect on MMP14 was mediated via PI3K pathway. Conclusions: The magnitude of insulin effects on MMP14 expression paralleled the amount of insulin receptors present on the respective placental cells. The control of insulin effects on the placenta undergoes a developmental shift from mother to fetus enabling fetal insulin to regulate placental development and function at the end of gestation. Insulin-sensitivity of MMP14 expression only in the arterial, but not in the venous, EC may have implications for placental angiogenesis regulation.

1967/B346
Stage Specific Expression of SAS1R in Mouse Oogenesis.
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SAS1R (Sperm Acrosomal SLLP1 Receptor) is an oocyte metalloprotease and binding partner for the sperm acrosomal protein SLLP1. To study the molecule's ontogeny, SAS1R was localized in the neonatal, pubertal and adult mouse ovary [O, 1.5, 4, 7, 14, 28 and 56 days] using guinea-pig anti-recSAS1R serum on Bouin's fixed paraffin embedded sections using the HRP method. Staging defined primary and secondary follicles, respectively, as oocytes surrounded with a single, cuboidal granulosa layer, or with two or more layers. SAS1R protein was first noted in oocytes in bilaminar secondary follicles beginning with day 4 and in all larger secondary, antral and Graafian follicles. SAS1R was not detected in resting naked oocytes or in oocytes of primordial or primary follicles nor in non-germ cells of any stage. Indirect immunofluorescence of ovulated oocytes and cultured embryos localized SAS1R in the ooplasm of germinal vesicle oocytes and on the oolemma of the microvilli domain in M2 oocytes. SAS1R was subsequently detected in the perivitelline space and in punctuate regions of the oolemma in the zygote and early embryo, becoming virtually undetectable in blastocysts. Since SAS1R expression was initiated in follicles with two or more layers of granulosa cells, the protein might be useful as a biomarker for follicular staging, particularly granulosa proliferation, or as a target for a reversible contraceptive that might act selectively on developing oocytes while sparing primordial and primary oogonia. Supported by NIH HD055129 and 5D43TW000654 from the Fogarty International Center.

Signal Transduction in Development II (1968 -1984)

1968/B347
Ectoderm-derived Sphingomyelin Signals Regulate Somitogenesis and Primary Myotome Formation in Chicken Embryos.
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Sphingomyelin (SM) produces several signaling lipids and is abundant in ectoderm and endoderm layers in chicken embryos but is very low in mesoderm tissues. The role of ectodermal SM is unknown in the development of somites. We therefore investigated segmental plate mesoderm (SPM) somitogenesis, somite patterning and primary myotome formation in 2 day-old chicken embryos with ectoderm exposed, in ovo, to neutral sphingomyelinase (bSMase) at 100-200 mU/mL for 6 hours. The reduction in SM was verified by lysenin toxin labeling. Ectoderm, untreated with bSMase, expressed variable lysenin-SM labeling with many large cell groups labeled extensively for SM while other groups either had numerous punctate SM micro-domains or showed an absence of SM except at cellular junctions. This pattern of ectoderm lysenin-SM labeling did not appear different between SPM and early somite areas. In cases of bSMase treatments, ectoderm cells enriched in SM were greatly reduced and coincided with an increase in cells showing punctate SM micro-domains and cells with SM only at cell borders. Furthermore,
bSMase addition did not affect somite segmentation, but periodically produced somites smaller or larger than normal size. Also, somites had normal dorso-ventral patterning. However, bSMase treatment caused a complete or partial loss of myotome formation in somites made from the SPM following overnight embryo growth. We conclude that a transition of SM occurs in ectodermal cells from those abundant in SM to those with minimal SM presence to suggest SM signaling of the segmental plate mesoderm for somite formation and primary myogenesis. NSF-IOS-0821324.

1969/B348
Identifying Endogenous PLC Isoforms Involved in the Signal Transduction Pathway at Bovine Oocyte Activation.
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Intracellular calcium (Ca^{2+}) release, a hallmark of oocyte activation, is the end result of a complex series of signal transduction pathways which have yet to be completely characterized in the bovine model. It is well known that src family kinases (SFK) activate Phospholipase C (PLC) which converts phosphatidyl inositol (4,5)-bisphosphate into diacylglycerol and 1,4,5-inositol trisphosphate (IP_3) which is directly involved in releasing Ca^{2+} from the endoplasmic reticulum. A two prong approach was undertaken to identify the mechanisms involved in the signal transduction pathway resulting in bovine activation. The first approach utilized immunoblotting to identify the presence of endogenous PLC isoforms in total bovine oocyte lysate using primary antibodies directed against four distinct isozymes of PLC. Immunoblotting was performed according to standard laboratory protocols and confirmed the presence of the PLC isoforms δ_1, δ_3, δ_4, γ_1, β_3 and β_4. The second approach involved microinjecting primary antibodies of ten different PLC isoforms into In Vitro matured bovine oocytes at a 1:100 dilution. Following microinjection, oocytes were fertilized and cultured In Vitro according to standard laboratory procedures (Reed et al., 1996). Activation and development were assessed by recording cleavage at 48 hours post fertilization. Microinjection of several PLC-specific antibodies resulted in no effect on cleavage rates while others significantly blocked development. Determining the presence and involvement of these intracellular signaling molecules in bovine oocyte fertilization and activation will result in better activation protocols for nuclear transfer (NT). Activating NT embryos in a more biological manner would ultimately improve the efficiency of the NT process along with facilitating study of specific aspects of activation by identifying messages associated with the process.

1970/B349
Substratum Compliance Modulates Tgf-B1-Induced Myofibroblast Transition.
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We examined the effects of varying substratum compliance (pliability) In Vitro on Transforming Growth Factor beta-1 (TGF-β1)-induced differentiation of corneal fibroblasts to myofibroblasts. TGF-β1 is a key signaling molecule involved in the fibroblast differentiation pathway. In vivo, these cells are localized to wound sites, areas in which they receive multiple cues provided by the surrounding tissue, possibly affecting their responsiveness to TGF-β1 signaling. Rabbit corneal fibroblasts were plated on polyacrylamide gels with Young’s moduli of 30 kPa or 100 kPa or on standard planar tissue culture plastic (TCP), then cultured with TGF-β1 at 0 ng/ml, 1 ng/ml, or 10 ng/ml concentrations. After 72 hours, RNA was harvested from all samples for quantitative real-time PCR analysis of the myofibroblast phenotypic marker α-smooth muscle actin (α-SMA). Cells grown on TCP without TGF-β1 treatment expressed negligible levels of α-SMA; cells treated with 1 or 10 ng/ml TGF-β1 expressed α-SMA at approximately 5- or 20-fold, respectively, over that of nontreated cells. However, preliminary data indicate that cells cultured with 1 ng/ml TGF-β1 and grown on 30 kPa surfaces, which are in the compliance range of the fibroblast-proximal structural
environment in vivo, expressed a 2-fold increase in α-SMA compared to similarly treated cells on TCP. Cells grown on surfaces of 100 kPa, which are above the physiological range of compliance, resulted in similar α-SMA expression levels to those on TCP over all concentrations of TGF-β1 treatment. These data suggest that the biophysical cue of compliance modulates differentiation of corneal fibroblasts to myofibroblasts.

1971/B350
Pkc Isoenzymes in Murine Embryonic Stem Cell Differentiation to Cardiomyocytes.
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The Protein kinase C (PKC) family of serine/threonine kinases have been shown to be involved in embryonic stem cell (ESC) differentiation to cardiomyocytes. However, the role of these isoenzymes in this process remains to be determined. Murine ESCs were differentiated to cardiomyocytes by the hanging drop method. Embryoid bodies were treated with PKC isoenzyme specific peptide modulators as of the sixth day of the differentiation process. Specifically, inhibition of βIIIPKC resulted in an increase in beating foci as well as mRNA for MLC2v and αMHC, indicating a role for this isoenzyme in ESC differentiation to cardiomyocytes. Subcellular distribution of the different PKC isoenzymes throughout differentiation was determined by confocal microscopy. αPKC was present during days 7-15 of the differentiation process in α-actinin positive cells, and its expression decreased with time. βIIIPKC was expressed in the nuclei of α-actinin positive cells, and was no longer expressed at day 16th of differentiation. βIIIPKC expression was concentrated adjacent to the nuclei throughout the differentiation process. εPKC was present in α-actinin positive cells during the differentiation process. However there was a change in the subcellular localization of εPKC during the differentiation process. εPKC was localized at the cytoplasm from day 7-9 of the differentiation process translocating to Z bands of α-actinin at day 10. εPKC remained at the Z bands throughout day 15. Translocation of εPKC is indicative of a change of function of this isoenzyme throughout differentiation; in fact treatment of ESC with a εPKC isoenzyme specific activator, from day 6-15 of the differentiation process increased the number of beating foci throughout day 10. from day 11-15 there was a decrease in the number of beating foci. As has been demonstrated in neonatal cardiomyocytes εPKC could be inhibiting spontaneous contractility from day 11 on. Modulating different PKC isoenzymes throughout the differentiation process and determining subcellular localization of different PKC isoenzymes may aid us to elucidate the role of specific PKC isoenzymes during ESC differentiation to cardiomyocytes.

1972/B351
A Mutation in Med12/Trap230 Increases Neuronal Sensitivity to Embryonic Ethanol Exposure in Zebrafish.
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Embryonic exposure to alcohol can result in a wide range of neuronal defects. The severity of these defects is dependent upon the ethanol concentration, length, and developmental timing of exposure. Additionally, genetic and environmental influences may alter the effects of ethanol exposure on brain development have been observed. Four transgenic lines were used in these studies: Tg(-1.8gsc:GFP)ml1; Tg(pax2a:GFP)e1; Tg isl1:GFP)rw0; and Tg(elavl3:EGFP)zf8. In addition, kohtalo (ktoy82) mutant zebrafish, which have a point mutation in the mediator complex gene med12, were crossed to these transgenic fish. Med12, alternatively known as Trap230, is a component of the mediator complex, and mutations in this gene can have profound effects on brain development. In transgenic, homozygous ktoy82 mutant embryos, the number of EGFP-
expressing neurons is significantly reduced as compared to their wild type siblings. Intriguingly, several features of med12 mutations are reminiscent of embryonic exposure to alcohol. In order to determine the effects of ethanol on brain development and to test for potential interaction or overlap between mutations in med 12 and ethanol exposure, wild-type and ktoy82 mutant transgenic embryos were treated with ethanol concentrations ranging from 0.5%-2.0% at mid-blastula, 11-25 somite stage, 24 and 36 hours post-fertilization (hpf) for various time periods. Ethanol treatment of wild type fish at later stages slightly disrupted only certain subsets of EGFP-expressing neurons without a significant overall effect. However ethanol treatment of Ktoy82 mutants, already somewhat deficient in these EGFP-expressing neurons, resulted in their near-elimination.

1973/B352
Role of Flotillins in Axon Guidance during Zebrafish Development.
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Flotillins are intracellular scaffolding proteins associated with the cytoplasmic face of lipid raft microdomains. They co-cluster with GPI-linked proteins, participate in the assembly of intracellular signaling complexes, and have been implicated in multiple signaling events, including Src signaling and cytoskeletal regulation by RhoGTPases. Flotillins are upregulated in neurons after injury and affect the ability of axons to regenerate. However, nothing is known about the functions of Flotillins during normal axon development. We are investigating potential roles of Flotillins during axon guidance in the developing zebrafish embryo. Zebrafish have three Flotillin genes, and of these only two are expressed in neurons - flotillin1a (flot1a) and flotillin2 (flot2). Flot1a expression is restricted to the nervous system, whereas flot2 is expressed in the nervous system, somites, and head mesoderm. We have used morpholino knockdown to study the functions of both Flot1a and Flot2. In addition, we are analyzing an insertional mutant for flot2, which is a likely loss of function mutation. Preliminary results suggest that morpholino knockdown of Flot1a leads to a reduction in contact repulsion between peripheral sensory axons. Moreover, flot2 mutants show possible defects in motor neurons, including axon stalling or ectopic branching. These observations suggest a potential role for Flotillins in axon development, which we will continue to test in future experiments.

1974/B353
Characterization of the Protein Complex (Es) Initiated By the Intracellular Domain of Individual Notch Paralogs.
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Notch protein is a family of evolutionary conserved single-pass transmembrane receptors that play an essential role during embryonic development and in the maintenance of tissue homeostasis in adult. There are four members of this family in vertebrates, Notch 1-4, that share similar structural motifs and similar maturation and activation processes. Most biochemical and structural studies that investigated molecules involved in the Notch signaling pathway used Notch1 as the prototype and extrapolated the findings to the other three Notch paralogs. However, genetic studies have revealed paralog-specific functions and biochemical studies have shown that individual paralogs have different activation strengths at given promoters. Therefore, individual paralogs likely have unique interacting protein(s). In the present study, we took several approaches to characterize and compare the protein complexes formed by the intracellular domain of individual Notch paralogs. Firstly, by using size exclusion chromatography, we found that the elution profiles of N1ICD (Notch1 intracellular domain), N2ICD, and N3ICD are similar but are distinct from that of N4ICD, indicating the N4ICD protein complex is different from the others. Secondly, using bioinformatics, we found the difference in the size of the N4ICD complex to that of the other three paralogs is likely the result of changes in several key amino acids involved in
interaction with MAML proteins. This may cause the core NICD/RBP-Jκ/MAML protein complex to become unstable. Finally, we are using fluorescent recovery after photobleaching and fluorescence correlation spectroscopy to study the dynamics of the NICD complex. To our surprise, our preliminary data suggests that GFP-tagged Notch intracellular domain does not bind, or only loosely binds, any immobile structure, such as chromatin. These results provide, in part, the molecular basis of the paralog-specific functions.

1975/B354

**Β-Parvin Regulates Cell Movements during Xenopus Gastrulation.**

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Temporal and spatial modulation of integrin α5β1 activity is required for the cell movements that characterize *Xenopus* gastrulation. Several lines of evidence indicate that these changes in integrin activity require the cytoplasmic domains of both the α and β subunit. Here we characterize *Xenopus* β-parvin, part of the PIP (PINCH, ILK, Parvin) complex, known to regulate integrin function through interactions with the β integrin subunit cytoplasmic domain. β-parvin is expressed at all stages of development and in situ hybridizations localize transcripts to the dorsal side of the embryo following fertilization. β-parvin is found in the dorsal mesoderm during gastrulation and in somites and the anterior pharynx following neurulation. In gastrula stage *Xenopus* embryos β-parvin co-precipitates with ILK and PINCH indicating an active PIP complex is present in the embryo. β-parvin is a scaffolding protein mediating protein complex formation through two calponin homology (CH) domains. Expression of either the CH1 or CH2 domain inhibits fibronectin (FN) matrix assembly during embryogenesis. Expression of the CH1 domain has no effect on activin-induced cell adhesion to FN substrates. However, in tissue explants the first CH domain is instructive for convergent extension even in the absence of a fibronectin matrix. These results indicate that the CH1 domain mediates signaling downstream of integrin adhesion to FN. A β-parvin construct containing only the CH2 domain inhibits activin-induced embryo cell spreading, persistent lamellipodia protrusions, and migration on FN substrates. In cultured A6 cells GFP-tagged RP2 localizes to focal adhesions. These results indicate that the CH domains of β-parvin act to link divergent integrin mediated signaling pathways during *Xenopus* embryogenesis.

1976/B355

**Perturbation of Mouse Retinal Vascular Morphogenesis by Anthrax Lethal Toxin.**

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Lethal factor, the enzymatic moiety of anthrax lethal toxin (LeTx) is a protease that inactivates mitogen activated protein kinase kinases (MEK or MKK). In Vitro and In Vivo studies demonstrate LeTx targets endothelial cells. However, the effects of LeTx on endothelial cells are incompletely characterized. To gain insight into this process we used a developmental model of vascularization in the murine retina. We hypothesized that application of LeTx would disrupt normal retinal vascularization, specifically during the angiogenic phase of vascular development. By immunoblotting and immunofluorescence microscopy we observed that MAPK activation occurs in a spatially and temporally regulated manner during retinal vascular development. By immunoblotting and immunofluorescence microscopy we observed that MAPK activation occurs in a spatially and temporally regulated manner during retinal vascular development. By immunoblotting and immunofluorescence microscopy we observed that MAPK activation occurs in a spatially and temporally regulated manner during retinal vascular development. By immunoblotting and immunofluorescence microscopy we observed that MAPK activation occurs in a spatially and temporally regulated manner during retinal vascular development. Intravitreal administration of LeTx caused an early delay (4 d post injection) in retinal vascular development that was marked by reduced penetration of vessels into distal regions of the retina as well as failure of sprouting vessels to form the deep and intermediate plexuses within the inner retina. In contrast, later stages (8 d post injection) were characterized by the formation of abnormal vascular tufts that co-stained with phosphorylated MAPK in the outer retinal region. We also observed a significant increase in the levels of secreted VEGF in the vitreous 4d and 8d after LeTx injection. In contrast, the levels of over 50 cytokines other cytokines, including bFGF, EGF, MCP-1, and MMP-9, remained unchanged. Finally, co-injection of VEGF-neutralizing antibodies significantly decreased LeTx-induced neovascular growth. Our studies not only reveal that MAPK
signaling plays a key role in retinal angiogenesis but also that perturbation of MAPK signaling by LeTx can profoundly alter vascular morphogenesis.

1977/B356
*Reactive Oxygen Species Derived By NADPH Oxidases Are Involved in Chondrogenesis.*
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Chondrogenesis is the process by which ectodermal mesenchymal cells differentiate into chondrocytes. Mouse embryonal carcinoma-derived cell line ATDC5 is a model of chondrogenesis in the early stages of endochondral bone development in response to insulin stimulation. Insulin-stimulated generation of H2O2 is crucial for insulin signal transduction. Although studies have shown that insulin in some non phagocytic cells induces reactive oxygen species (ROS) generation, little is known about the role of ROS in chondrocytes. We demonstrated that generation of ROS is increased during chondrogenesis using ATDC5 cells. And the treatment of antioxidant enzymes, N-acetylcysteine (NAC) and diphenylen iodonium chloride (DPI) suppressed the differentiation. ROS derived from NADPH oxidase (NOX) homologues have been suggested to regulate various physiological and pathological processes. We investigated whether ROS generated by NADPH oxidases is involved in chondrogenesis. Among NOX family, NOX1, gp91phox (NOX2) and NOX4 were highly expressed during differentiation of ATDC5 cells. Suppression of NADPH oxidases using siRNA inhibited ROS generation, chondrogenic differentiation and induced apoptosis of ATDC5 cells. These data suggest ROS generated by NADPH oxidaes is involved in early chondrogesis usign ATDC5 cells.

1978/B357
*Noggin Secretion Regulation by Rab3d Is Essential for Xenopus Anterior Neurulation.*
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Rab3d is a member of Ras-related small GTPase family of secretory Rab, Rab3. In this study, we showed that Xenopus Rab3d is expressed specifically in the anterior neural ridge (ANR) at neurula stage when neural plate converges and folds to form neural tube. Morpholino (MO) mediated depletion of Rab3d resulted in neurulation defects both in neural plate convergence and folding. Interestingly, perturbation of BMP signaling by dominant negative Smad1 rescued Rab3d-MO mediated neurulation defects, suggesting that Rab3d inhibits BMP signaling during neurulation. Because Rab3d is known as a secretory Rab, we tested secretions of BMP antagonists, Noggin and Chordin. Western blot analysis of secreted proteins from Rab3d-MO injected animal caps showed that Rab3d specifically regulates secretion of a BMP antagonist, Noggin but not Chordin in a caffeine dependent manner. We also showed that Rab3d is co-localized and co-immunoprecipitated with Noggin. These findings suggest that Rab3d-mediated secretion regulation of a BMP antagonist, Noggin, is one of the mechanisms of Ca2+ triggered anterior neurulation.

1979/B358
*Varied Roles for Primary Cilia on Shh Producing and Responding Cells of the Limb Bud.*
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Primary cilia are immotile microtubule based structures found on nearly all cells of the mammalian body and are formed by a process called intraflagellar transport (IFT). Disruption of ciliogenesis through mutations in IFT genes including IFT88 (Polaris) results in early embryonic lethality. In Vivo and In Vitro analyses of the phenotypes seen in cilia mutants indicate that this organelle has a critical role in regulating several signaling pathways, including hedgehog and wnt, both of which are important for normal development, differentiation, and cell fate determination. Using IFT88 mutant limb bud cultures we previously found that cells lacking cilia are unable to
respond to sonic hedgehog (Shh) and have defects in activation and processing of the hedgehog responsive transcriptional regulators Gli2 and Gli3, respectively. In addition, many of the Shh pathway components including Patched, Smoothened, Suppressor of Fused, Kif7, and the Gli proteins, have been shown to localize to the cilium. To further analyze the function of cilia in Shh signal transduction and development, we are using a conditional allele of IFT88 and transgenic Cre mouse lines to disrupt cilia in Shh producing or responding cells. Surprisingly, we have found that the loss of cilia on the Shh producing cells of the mouse is not lethal, does not impact digit number, but does seem to affect bone development. This is in contrast to the extensive polydactyly seen mice lacking cilia on the cells throughout the limb mesenchyme. Analyses of the Shh responding cells is revealing varied phenotypes ranging from polydactyly to endochondrial bone formation defects depending on when the cilia are disrupted. Together these data support the hypothesis that cilia on the different populations of cells in the developing embryo will have varied roles and the impact of their loss is temporally dependent.

1980/B359
Lipid Binding and Activation of Src in Xenopus Laevis Sperm.
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Based on phosphatidic acid (PA) mass measurements and that other reports suggesting a role for Src, our current model for Xenopus sperm acrosome reaction may involve an increase in PA and this lipid would bind and activate Src. Sperm suspensions were exposed to: phosphatidic acid (PA), jelly water (which contains the agonist ARISX that induces the Xenopus sperm acrosome reaction; see work of Iwao), calcium ionophore Ionomycin or different concentrations of Modified Barth’s Saline (MBS). Sperm suspensions exposed to 400uM PA (10 s) showed a significant drop in Src activation (as measured by tyr 418 phosphorylation). When exposed to jelly water, PP60Src activity increased. Since decreased tonicity can activate Xenopus sperm (resulting in increased sperm motility), we note that in 15% MBS the active Src levels didn’t drop significantly until 20 minutes (37% decrease from baseline) while the 33% MBS had a significant drop after 10 minutes (12% decrease). This would suggest that Src activity would be not be associated with the start of sperm activation. Furthermore, we were unable to detect any changes in phosphoSrc in activated sperm (30 seconds, 33% MBS) and inactive sperm (100% MBS). Finally, to quantify the interaction between PA and Src, we have modified published versions of the lipid protein vesicle sedimentation assay. We are obtaining binding data with PA, but the apparent Kd for a control protein (BSA) binding to PC/PA vesicles was 0.0171, that for BSA binding to PC liposomes was 0.0264 and the apparent Kd for BSA binding to PC/LPA liposomes was 0.0321.

1981/B360
Musashi 1 Relocates to the Nucleus in Differentiating Neural Cell Lines.
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Musashi was originally identified in drosophila and has two mammalian forms (Msi1, Msi2). Msi1 has been identified as an RNA binding protein having a role in stem cell self-renewal, is expressed in some cell cancers and also in the retina. The stem cell pathway is by repression of the Numb protein, which is required for cell cycle exit and differentiation. One potential role of musashi is to repress the specifically bound RNA translation and therefore regulate protein expression. The bound RNA targets include known cell cycle and differentiation regulators including Numb and p21WAF. In this current study we have immunochemically identified the intracellular localization of Msi1 in neural cell lines using a monoclonal antibody (Abcam Cat# ab52865). Neural cell lines from both rat (PC12, B35) and mouse (C17-2, HT4, RGC5) were grown in tissue culture using existing protocols, replated onto collagen-coated coverslips and incubated in media with or without the addition of 1 mM dibutyryl cyclic AMP (dbcAMP, differentiation media). After 3 days all cells were fixed, labelled with Msi1 antibody and the localization pattern (cytoplasmic/nuclear) identified. Msi1 was identified as mainly cytoplasmic in

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all the undifferentiated neural lines. Treatment with dbcAMP lead to morphological changes in some cell lines (expression of elongated cell processes) and a shift to the nuclear staining pattern. In C17-2 and HT4 neurons, nearly all treated cells were nuclear positive and cytoplasmic negative. Both the B35 and the PC12 cells showed a shift to a nuclear localization. RGC5 cells showed a mixed distribution pattern in both conditions. Other retinal studies have shown both a developmental and differentiated expression pattern for Msi1 in retinal ganglion cells. These results are consistent with the concept that exposure of the Msi1 nuclear localization sequence, found in the first RNA-binding sequence, leads to a nuclear translocalization. Removal of cytoplasmic Msi1 would then allow translation and expression of Numb, allowing neural cell line exit from the cell cycle and neural differentiation.

1982/B361
The Role of the 'Multitalented' Jun N-Terminal Kinase (JNK) Signalling Pathway in Neuronal Morphogenesis.
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Understanding how signalling molecules regulate various aspects of cell morphology is a fundamental question in neuroscience. One confounding factor towards a complete understanding of the topic is the constant reiteration of the same proteins involved in various aspects of neuronal morphogenesis; how can the same molecule regulate so many aspects of cell morphology? The Jun N-terminal kinase (JNK) pathway, typifies such a pleitrophic factor and in neurons has been implicated in axon polarity, extension, dendrite formation and synaptic growth. Here we show that two parameters are critical in defining JNK-dependent axonal stability: signal strength and signal duration. Using Mushroom Body (MB) neurons in the *Drosophila* brain as a model, our data shows that complete loss of the *Drosophila* JNK Basket (Bsk) results in axon degeneration. *Drosophila* JNK is activated by phosphorylation of two Jun N-terminal Kinase Kinases (JNKKs): Hep and MKK4. Surprisingly loss of either JNKK gave a contrasting axon overextension phenotype. We resolved this conundrum by demonstrating that it is the partial inactivation of Bsk signals that results in axon overextension. This suggests that Bsk signals safeguard against neurodegeneration at lower thresholds and prevent axon overextension at higher levels. We also demonstrate that sustained Bsk signals are essential throughout the development of MB neurons to maintain proper axonal morphogenesis but are dispensable at adulthood. This suggests a change in requirements for JNK signalling between the developing and adult brain. Finally we present evidence demonstrating that the AP-1 transcription factor complex regulates axon stability downstream of Bsk. Weak AP-1 signals are able to protect against neurodegeneration, while stronger AP-1 signals prevent axon overextension. We therefore propose that graded Bsk inputs are translated into AP-1 transcriptional outputs consisting of Fos and Jun proteins.

1983/B362
Patched Mobilizes Lipoprotein-Derived Lipids to Inhibit Smoothened.
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Hedgehog is a lipoprotein-borne ligand that signals by binding to Patched, reversing Patched-mediated repression of Smoothened signaling. Repression correlates with lower levels of Smoothened on the basolateral membrane, but how Patched regulates Smoothened trafficking and activity is not understood. We have shown that Patched sequesters internalized lipoproteins in endosomes and regulates, via its sterol-sensing domain, the lipid composition of this compartment. Smoothened traffics through Patched endosomes and is trapped there by mutations in the Patched sterol sensing domain. Patched uses lipids derived from lipoproteins to promote Smoothened degradation and reduce its ability to stabilize Ci155. However, other Patched dependent events are required to fully repress Smoothened signaling. Our data suggest
that Patched-dependent changes in endosomal lipid composition modulate the balance between Smoothened recycling and degradation and alter its signaling activity, and that Hedgehog may signal by regulating the utilization of the lipoproteins that carry it.

1984/B363
Receptor Tyrosine Kinase Signaling Interactions in Neuroblastoma Cell Lines.
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We hypothesize that receptor tyrosine kinases (RTKs) functionally interact to drive key cell fate decisions in neural crest, and associate with kinase substrates and downstream effectors in endosomes. To test this hypothesis, we performed PhosphoScan analysis (Nat. Biotechnol. 23:94, 2005) on cell fractions including purified endosomes from neuroblastoma cell lines (Traffic 10:938, 2009). Our approach is to 1) sort and identify proteins according to fold stimulation by ligand-induced receptor activation; 2) search for patterns and common pathways using overview tools such as hierarchical clustering, and 3) manually interrogate the data with an eye towards identifying RTK-activated signaling pathways, noting similarities and differences from different cell lines and different RTKs. We have focussed first on several different RTKs that have been identified by phosphotyrosine peptide analysis in cell lysates. As expected, EGFR is activated by EGF; TrkA by NGF. Other tyrosine phosphorylated RTKs (FGFR, PDGFR, InsulinR, DDR2) were also detected. Anaplastic lymphoma kinase (ALK) was highly phosphorylated in two neuroblastoma cell lines and was prominent in endosome fractions. Proteins not identified from lysates are revealed in organelles, for example c-Kit and Ret were identified exclusively in the endosomes of SMS-KCN cells. Akt (PKB) activation is particularly important for amplification of specific signaling pathways that affect programmed cell death and differentiation. We have employed Akt substrate analysis (similar to phosphotyrosine peptide analysis) from cell lysates and endosomes. Interestingly, the RTKs, ALK, EGFR, DDR2, EphA2, EphB1, EphB2, EphB4, FGFR1, FGFR4, InsulinR, c-Kit, PDGFRα, PDGFRβ, RDR2, Ret, and TYRO3 were all identified as Akt substrates in LAN-6 cells and their endosomes. Which RTK is the primary driver, and which are the effectors, of a signaling cascade? We hypothesize that ALK and TrkA receptor expression instructs cell fate decisions in neural crest through activation of specific signaling pathways, including pathways activated by intracellular signaling endosomes. Experiments are underway to determine whether there is trans-activation (or -deactivation) between ALK and TrkA.


1985/B364
Characterization of a Dorsalized and Cell Migration Maternal Effect Mutant in Zebrafish.
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Maternal factors are required for many early developmental processes including fertilization, egg activation, and formation of the body axes during early development of the zebrafish Danio rerio. Recently the lab has performed a recessive maternal-effect mutagenesis screen and identified a number of mutants with defects in the early developmental processes of egg activation and body axis formation. One such mutant, 24BDTH exhibits abnormal dorsal-ventral patterning, displaying a dorsalization of the embryonic axis. Clutches of embryos from 24BDTH mutant mothers are characterized by the five classic dorsalized phenotypic classes, as well as some additional defects. The additional defects appear to be the result of altered morphogenesis and defects in cellular migration of the blastoderm cells to surround the yolk. Additionally these embryos show an expansion of dorsal markers and a corresponding reduction in ventral markers during gastrulation which is indicative of dorsalization. 24bdth mutant embryos also appear to be
defective in convergence and extension. We mapped the 24BDTH mutation to chromosome 17, within a 8.4 MB interval and efforts are currently underway to positionally clone the affected gene.

1986/B365
An Unbiased Chemical Screening Approach to Dissect C-Met Signaling in EMT.
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The c-Met receptor initiates epithelial mesenchymal transition (EMT) in a number of different cell types, including cancer cells. During EMT, cells disassemble cell-cell junctions and migrate independently. How c-Met activation directs this change in cellular behavior remains unclear. Here, we attempt to dissect the c-Met signaling network at a systems level. In our approach, we attempt to identify compounds that inhibit the cells from responding to c-Met signaling by scattering, and then identify the molecular targets of those compounds using computational and biochemical approaches. From among 50,000 compounds, we identified 38 that prevent cells from undergoing EMT in response to c-Met activation. We have confirmed that a number of these drugs are capable of preventing EMT by blocking specific targets using unrelated inhibitors or dominant negative mutant expression. Our results implicate two major pathways in c-Met induced EMT. The first of these, the NF kappa B pathway, has already been documented to contribute to EMT via the c-Met receptor and provides proof-of-principle to our approach. The second pathway, which employs epithelial calcium channels, has not been previously tied to the c-Met receptor. Our ongoing efforts are to apply compound treatments to cells expressing constitutively active mutants of proteins that mediate c-Met signaling, thus determining the relative positioning of components within the c-Met signaling network.

1987/B366
The Role of Neurotrophins in Schwann Cell Precursor Migration.
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Schwann cells are peripheral myelinating glia found in the nerves of vertebrate organisms. Schwann cells originate from the neural crest, which are a highly migratory stem cell population very early in development. Schwann cells come from a special progenitor population in the crest referred to as the Schwann cell precursors (SCP). The aim of this research is to investigate the influence that neurotrophins may have on Schwann cell precursor migration. We used two methods to study the motility and chemoattraction of SCPs for neurotrophins. In the first was the wound assay on SCPs in the presence of neurotrophins. We counted the number of cells that invaded the wound after a 5hrs in order to determine enhancement of motility. The second assay utilized the chemotaxis chamber to test the directionality of the SCPs towards a source of neurotrophins. The number of cells that traveled through the gradient towards the neurotrophins was counted and served as an indicator of chemo-attraction. Our results show that of GDNF, EGF, MIF, NGF and Slit2, Slit 2 and GDNF enhanced the motility and EGF and GDNF were chemoattractants.

1988/B367
Endothelial Vinculin Expression Is Required for Normal Vascular Development.
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Vinculin (Vcl) is a ubiquitously expressed membrane-associated protein, linking the actin cytoskeleton to the sarcolemma. Vcl is found in cell-matrix and cell-cell contact sites. Global deletion of Vcl caused embryonic lethality at embryonic day (E) 10.5, due to cardiac and neurological defects. Fibroblasts isolated from mutant embryos showed reduced matrix adhesion and a higher migration rate. We generated floxed Vcl (Vcl²⁰⁰) mice and previously showed cardiac-myocyte specific excision caused death at young age. To investigate Vcl’s role in
vascular development we conditionally ablated Vcl in endothelial cells (EC) of VCL^{fl/fl} mice using
Cre driven by promoter of the murine endothelial receptor tyrosine kinase (Tie2-Cre, Jackson
Lab# 004128). Results: Vcl^{fl/+} Tie2-Cre+/- males were crossed with Vcl^{fl/fl} Tie2-Cre-/- females but
no Vcl^{fl/fl} Tie2-Cre+/- mice (eVclKO) were liveborn (5 litters, n=29, 34% Vcl^{fl/fl} Tie2-Cre-/-, 38%
Vcl^{fl/+} Tie2-Cre-/- and 28% Vcl^{fl/+} Tie2-Cre+/-). Vcl^{fl/+} Tie2-Cre+/- developed and bred normally.
Timed matings showed growth retardation of eVclKO embryos compared to controls starting at
E11 followed by death between E13.5-14. Mendelian distribution was normal until E13.5 (8 litters,
n=67, 25% Vcl^{fl/fl} Tie2-Cre-/-, 24% Vcl^{fl/+} Tie2-Cre-/-, 28% Vcl^{fl/+} Tie2-Cre+/- and 23% eVclKO). At
E11.5-12.5 eVclKO embryos showed fewer, less branched and spontaneously enlarged vessels,
compared to controls. By E13.5 generalized perivascular bleeding was noted in eVclKO, but not
in other genotypes. Given the bleeding, the importance of zonula occludens (ZO) proteins in
endothelial barrier function, and that Vcl deficient cardiac myocytes had abnormal distribution of
ZO-1, we examined whether Vcl and ZO-1 directly interact. Yeast two-hybrid protein interaction
screening confirmed direct binding of Vcl with ZO-1. Conclusion: Our findings demonstrate that
endothelial Vcl expression is crucial for normal vascular development. Investigation of Vcl's
function in EC migration, adhesion, survival, and proliferation, potentially through its interaction
with ZO1, will be explored in further studies using this unique model system.

1989/B368
Leukemia Associated RhoGEF and RhoA in Smooth Muscle Cell Regulation.
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The regulation of smooth muscle cell (SMC) differentiation is critical during vascular development,
and perturbations in this process contribute to a number of cardiovascular pathologies including
atherosclerosis, hypertension, and restenosis. We have shown that activation of RhoA by
sphingosine-1 phosphate (S1P) stimulates SMC-specific gene expression by promoting the
nuclear localization of the myocardin-related transcription factors (MRTFs). The goal of the
current study was to identify the signaling pathway by which S1P regulates RhoA in SMC. Using
a combination of receptor-specific agonists and antagonists we identified S1P2 as the major S1P
receptor sub-type that regulates SMC-specific promoter activity and differentiation marker gene
expression in primary SMC cultures. In addition, over-expression of Gα12 or Gα13 also increased
SMC specific transcription, a result in excellent agreement with the known G-protein coupling
properties of S1P2. Given previous studies on the interaction of Gα12/13 with the RGS subfamily
of RhoGEFs (LARG, PRG, P115), we hypothesized that one or more of these RhoA activators
was important in S1P-mediated SMC differentiation. While expression of each of the RGS
RhoGEFs activated SMC specific transcription, LARG exhibited the most robust effect invoking a
10 to 15 fold increase SM22 and SM α-actin promoter activity. LARG expression also resulted in
increased stress fiber formation and MRTF-A nuclear localization. Importantly, siRNA-mediated
depletion of LARG inhibited activation of RhoA by S1P and also inhibited the effects of S1P on
endogenous SMC differentiation marker gene expression and SMC specific promoter activity.
Finally, knockdown of LARG promoted SMC migration as measured by scratch wound and
transwell assays. Taken together our data indicate that stimulation of RhoA activity by S1P2-
dependent activation of LARG plays a critical role in the regulation SMC phenotype. Additional
examination of this signaling pathway should aid in the development of specific and efficacious
treatments for cardiovascular disease.

1990/B369
Analysis of Proteomic Profiles during Embryonic Heart Development Using Two
Dimensional Differential Gel Electrophoresis (2D-Dige).
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Morphogenesis of the heart is a transformation from a single tube to a four-chambered complex
structure. Key to the understanding of the dynamic morphological transformation is an unbiased
profiling of gene expression and protein signaling. Due to its small size and accessibility, the
application of gene/protein profiling techniques such as DNA microarray and proteomics to the embryonic heart has been very limited. We attempted the use of the two dimensional gel electrophoresis (2DE) and mass spectrometry, and successfully obtained a proteomic profile (~350 spots) of the chick embryonic myocardial proteins. In particular, to be able to analyze expression levels quantitatively during development, we used a novel fluorescent differential gel electrophoresis (DIGE) protocol. Hearts at two developmental states, stage 29 (day 6 in 21 day incubation period) and stage 36 (day 10), were lysed and labeled with two fluorescent dyes (Cy 3 and Cy5), followed by isoelectric focusing and SDS-PAGE in a single gel. Signal intensity values of individual protein spots were normalized to the internal control which is equally mixed samples labeled by Cy2. This method basically removes variability between gels, which has been a major issue with 2DE. Among ~350 proteins, we have identified approximately 17 up-regulated and 20 down-regulated proteins from stage 29 to stage 36. Up-regulated proteins included aldolase-C and 3-hydroxybutyrate dehydrogenase, both of which have been implicated in metabolic pathways in the myocardium. Our data demonstrate that the use of 2DE and DIGE is highly applicable to obtain an unbiased protein expression profile which will be highly informative to understand heart morphogenesis.

1991/B370
The Role of Autophagy in Diabetic Embryopathy.
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During the developmental progression from oocyte to embryo, maternal proteins and RNAs are degraded and quickly replaced by newly synthesized embryonic constituents. Recent studies have concluded that autophagic degradation within early embryos is essential for the breakdown of these proteins leading to normal preimplantation development. The objective of this study was to determine whether autophagy was altered due to metabolic milieu of diabetes in preimplantation development leading to the development of late stage diabetic embryopathy. Methods: for In Vivo studies, diabetes was artificially induced in B6SJL mice by streptozotocin treatment. In Vitro studies were carried out in high (52mM) glucose conditions. for embryo retrieval mice were super-ovulated by standard hormone injections and uterine horns were flushed at the appropriate time after mating. Standard procedures of electron microscopy (EM) and immuno-fluorescence were used. Also, microanalytic techniques to measure enzyme activity of single cells were employed. Results: Our results indicate that a maternal diabetic environment, In Vivo or in vitro, triggers an increase in autophagy. First, diabetic conditions triggered an increase in GAPDH activity suggesting an increase in autophagy. These conditions also led to a decrease of Lactate Dehydrogenase activity signifying an increased degradation of long lived proteins. Furthermore, oocytes obtained from diabetic mice displayed an increase in the number of autophagosomes by EM. Finally, GLUT8, the main glucose transporter during the blastocyst stage was found to translocate to the autophagosomal membrane during activation of autophagy. Conclusions: We conclude that the metabolic milieu of diabetes triggers an increase of autophagy during embryo development, which in turn leads to aberrant rates of protein degradation. We believe that accelerated or delayed degradation of key enzymes in development may be responsible for the poor developmental outcomes observed in offspring born to diabetic mothers.

1992/B371
The Essential Role of Cop9 Signalosome in Skeletogenesis.
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In a yeast two-hybrid screening, we identified transcriptional cofactor Jab1/Csn5 as a novel interacting protein of Runx2. Jab1 is the 5th component of the evolutionarily conserved COP9 signalosome (Csn5) that plays essential roles during various developmental processes by modulating the function of other transcription factors. In this study, we utilize loxP/Cre system to
delineate the specific roles of Jab1 in skeletogenesis. Loss of Jab1 specifically in chondrocytes with Col2a1-Cre led to lethal chondrodysplasia and severe dwarfism at birth in mice. In E18.5 mutant embryos, all the skeletal elements developed via endochondral ossification were all extremely small. In the mutant mice, the cells immediate adjacent to hypertrophic chondrocytes, the presumptive per-hypertrophic zone, were of much larger size and severely disorganized compared with control mice. Moreover, immunostaining revealed that those atypical large chondrocytes also ectopically expressed Runx2, a positive regulator of chondrocyte maturation. Interestingly, in our preliminary study, Jab1 also inhibited Runx2 transcriptional activity in cell culture. Thus, in Jab1 flox/flox; Col2a1-Cre mutants, chondrocyte maturation is likely to be defective at least partly due to up-regulated Runx2 expression. To investigate Jab1 function in early osteochondroprogenitor cells, we deleted Jab1 in the limb buds with Prx1-Cre mice. Jab1 flox/flox; Prx1-Cre mice displayed drastically shortened limbs and doomed skull and most of them died shortly after birth. Histological analysis revealed the absence of defined hypertrophy zone in the mutants. Immunostaining showed the ectopic expression of Sox9, a negative regulator of chondrocyte hypertrophy, in the mutant chondrocytes adjacent to bone marrows. Lastly, the few Jab1 flox/flox; Prx1-Cre mutant mice that survived into adulthood all exhibited extremely shortened limbs and decreased bone density. In conclusion, Jab1/Csn5 serves unique and distinct roles at successive steps of skeletogenesis and postnatal bone growth likely by regulating the stability and function of key transcription factors including Runx2 and Sox9.

1993/B372

The Effect of (-)-Epigallocatechin Gallate in 3t3-L1 Preadipocytes Development.
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Obesity is a chronic illness that may involve long term treatment. There is compelling evidence showing that subjects with obesity are at an increased risk for a variety of diseases, including type 2 diabetes and hypertension. In order to cure obesity, an important mean is to develop an agent that can reduce body fat and improve metabolic homeostasis. Tea is the second most widely consumed beverage in the world. In recent years, green tea has been gaining its popularity among the tea. It contains high content of (-)-epigallocatechin gallate (EGCG) which is considered as the chemopreventative agent of cancer, obesity, diabetes and cardiovascular disease. The purpose of this study is to evaluate the effect of EGCG in adipocyte development which contributes to obesity. 3T3-L1 preconfluent preadipocytes and postconfluent preadipocytes during maturation were incubated with physiological (0.1μM, 0.2μM, 0.5μM, 1μM) and pharmacological (5μM, 10μM) concentrations EGCG respectively for five days or eight days. The effect of EGCG on the proliferation of preconfluent preadipocytes to their differentiation into matured adipocytes were also examined by incubating preadipocytes with EGCG for thirteen days. Culture media containing EGCG were refreshed twice daily for the respective incubation periods. at the end of the experiments, cell numbers were counted with a cell counter (Model Z2, Beckman Coulter, Fullerton, CA) at day 6 and day 14. The lipid contents of matured adipocytes were quantified by staining the cells with Oil-Red-O. The results showed that EGCG at the dose of 10μM significantly reduced the cell numbers of preadipocytes at day 6. The same response was seen in cells incubated with EGCG for the full stage of adipogenesis. In addition, the lipid contents in adipocytes were also reduced by the same concentration of EGCG. This suggests that EGCG inhibits proliferation of preadipocytes and prevents its differentiation into matured adipocytes, thus reducing adipogenesis.

1994/B373

Role Notch Signaling Pathway in Ocular Lens Development and Pathology.
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The ocular lens comprises concentric layers of differentiated, amitotic fiber cells capped by a monolayer layer of immature, anterior epithelial cells. Canonical Notch signaling (CNSig) is activated when Notch ligands such as Jag1 bind to the extracellular domain of any of the four known Notch receptors on adjacent cells. Ligand binding leads to gamma-secretase (GS) cleavage of the Notch intracellular domain (NICD). The NICD translocates to the nucleus and binds the transcriptional repressor CBF-1 to activate target genes. CNSig in lens was previously demonstrated using a conditional knockout of CBF-1 (CBF-1CN) generated using the Pax6-Le-Cre promoter to ablate CBF-1 expression in lens. CBF-1CN mice were microphthalmic, indicating that CNSig is required for lens epithelial cell proliferation. We recently observed that the N2ICD accumulated to high levels in post-mitotic differentiating rat lens epithelial cells (RLEC) In Vivo as well as in RLEC induced to differentiate ex vivo using FGF. Active Notch2 signaling was required for proper expression of differentiation markers N-Cadherin, p57Kip2 and Jag1 during FGF induced differentiation of neonatal RLEC. Therefore, we used the Pax6-Le-Cre promoter to generate a conditional knockout of the Notch2 receptor (Notch2CN). Notch2CN mice were microphthalmic, indicating that signaling through Notch2 is required for the previously observed effect on lens proliferation in CBF-1CN mice. Notch2CN lenses lacked epithelial cells and fiber cells were disorganized and failed to elongate normally. To determine if CNSig has a role in pathology of the lens such as Posterior Capsule Opacification (PCO), a severe side effect of cataract surgery, we examined the effect of inhibiting CNSig on the key marker of PCO, alpha-smooth muscle actin (alpha-SMA), using GS inhibitors. Inhibition of CNSig caused an almost complete suppression of TGF-beta induced alpha-SMA mRNA in human lens epithelial cell line FHL-124 as determined by RT-PCR and protein level of alpha-SMA in RLEC as determined by immunofluorescence. These results demonstrate that Notch2 signaling is essential during ocular lens development and is important for some aspects of lens pathology, PCO.

1995/B374
Regulation of β-Actin Expression in Neonatal Rats Subsequent to Prenatal Nicotinic Exposure.

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A frequently used method to detect and analyze differential protein expression in samples is Western immunoblotting of tissue homogenates or cell extracts. Quantitative measurements are subject to error due to uneven loading of samples or over-reliance on normalization. Therefore an internal loading control like α-tubulin, β-actin or GAPDH is often necessary for comparison because the levels of these proteins are believed to remain constant across tissues and species. Little information is available concerning the stability of their expressions under experimental conditions although many papers have reported differential actin expression upon drug treatment or in certain diseases. Here, we report that in response to prenatal nicotinic exposure, postnatal day 1 (P1) animals show increased expression of β-actin in the developing hippocampus, while other internal controls were not changed with nicotine. This study was carried out using Sprague Dawley rats at P1, P14 and P63, previously exposed to nicotine for 14 days from gestational day 7 (G7) to G21 and then compared to saline-exposed age- and sex-matched controls. Equivalent protein from hippocampus was loaded onto SDS-PAGE gels, blotted, and probed with antibodies specific for α-tubulin, β-actin, or GAPDH as well as other differentially expressed synaptic markers. We demonstrated that prenatal nicotinic exposure induced a significant increase in β-actin expression at postnatal day 1 (16%, P<0.01). This regulation was similar to other synaptic proteins studied at the same age, but not with the internal controls GAPDH and α-tubulin. The difference in β-actin expression between nicotine and saline treated groups disappeared at adolescent and adult ages. Our study suggests that β-actin whose expression increased following prenatal nicotinic exposure is not always a suitable internal control for experimental conditions. To ensure the accuracy of data claiming up- or down-regulation, β-actin must be evaluated in conjunction with other controls such as, GAPDH and α-tubulin.

1996/B375
Teratogenic Effect of Low Doses of Nicotine on Early Avian Development.
Nicotine is one of the major teratogenic chemicals that cross the placenta in smoking pregnant women. Previous teratogenic studies in animals used nicotine levels exceeding those measured in human amniotic fluid of smoking mothers and were conducted at relatively late stages of embryonic development. Here, we injected physiologically relevant amounts of nicotine (10, 100, or 1,000 nM) into the air sac of chicken eggs at 24 hours of development to determine nicotine's teratogenic effects in younger embryos. Specimens were collected and examined 4 days after a single nicotine injection. Embryos exposed to 100 or 1,000 nM nicotine were significantly smaller, as evidenced by a decrease in wet weight and crown rump length, and exhibited a higher frequency of gross morphological abnormalities such as anencephaly. 1,000 nM nicotine also caused delayed developmental progression. Embryos treated with 10 nM nicotine were unaffected. We also used immunohistochemistry to analyze the expression of nicotinic acetylcholine receptors (nAChRs) in very early embryos. We found expression of the $\alpha_{15}$ nAChR subunits in developing myotome as early as 3 days, and expression of the $\beta_2$ nAChR subunit in pharyngeal arch tissue at 2 days and in neural tissue by 3 days. Our data show that very young embryos express nAChRs, and that a single exposure to a low dose of nicotine increases the risk of congenital defects and causes a significant decrease in embryonic growth rate.

**1997/B376**

**Identifying Transcription Factors Regulating Touch Sensory Neuron Development.**

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Peripheral somatosensory neurons can be classified based on their sensory modalities. Each neuron selectively responds to and transmits one of the following four types of stimuli: thermal, painful, touch, or proprioceptive. Significant progress has been made in understanding transcription programs that specify nociceptive (pain and heat) and proprioceptive sensory neurons. However, factors that lead to the specification of touch sensory neurons or mechanoreceptors are largely unknown. To find candidate genes involved in this process, we performed microarray-based analyses to identify genes preferentially expressed in the maxillary division of the trigeminal ganglion in mice, because maxillary trigeminal neurons are enriched in whisker-innervating touch neurons. Through this screen, we found a set of transcription factors as candidates for determining mechanosensory neuron fate. Conditional deletion of one of them lead to the drastic reduction in the number of TrkB-positive mechanosensory neurons. We are currently carrying out detailed molecular and anatomical analyses of the conditional mutant mice.


**1998/B377**

**Epidermal Patterning by Protein Depletion Mechanism in Arabidopsis Thaliana.**

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Intercellular communication through protein movement is frequently employed in developmental processes in plants. During trichome patterning on leaves in Arabidopsis thaliana plasmodesmatal based protein movement is relevant to inhibit trichome formation. Theoretically, patterning can be achieved by either active inhibition or by the removal of trichome promoting activity. The Arabidopsis WD40 repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) is part of the trichome promoting complex and seems to be involved in lateral inhibition through its removal in trichome neighboring cells. Whereas the TTG1 gene expression is quite homogenous, the protein accumulates in trichome initials and is depleted in the neighboring cells. Previous work in our group showed that TTG1 is a mobile protein that can move into trichomes. Here we
address the question how TTG1 protein is redistributed. We show that TTG1 is sequestered in
the nucleus of trichomes and that its subcellular localization is greatly influenced by the bHLH
transcription factor GLABRA3 (GL3), which is part of the trichome activator complex. GL3
expression pattern, subcellular localization and molecular interaction data provide a strong basis
to support it as a potential attractor of TTG1 into trichome cells and thereby leading to TTG1
depletion in the neighboring cells. Trichome rescue experiments by tissue specific expression of
GL3 and/or TTG1 further supports that GL3 attracts and greatly influence the mobility of TTG1.

1999/B378
A Role for the Ring Finger Domain of the Peroxisomal Membrane Protein PEX2 in
Photomorphogenesis in Arabidopsis.
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Arabidopsis plants display two contrasting developmental growth forms in dark and light. In dark,
plants undergo skotomorphogenesis by having a long hypocotyl and an apical hook with small,
folded, and pale cotyledons. In contrast, light-grown plants are photomorphogenic in that they
typically exhibit the reverse phenotype of that in the dark. The phenotype in dark-germinated
seedlings is due to the concerted role of COP/DET/FUS proteins, which form a protein complex
that targets the positive regulators of photomorphogenesis, eg. HY5, HYH, LAF1, for proteolysis
via the proteasome. The det1 plants have a photomorphogenic phenotype in the dark, suggesting
the inability of the DET1-associated complex to tag the positive regulators of
photomorphogenesis for degradation. The gain-of-function mutant ted3 (reversal of det1) was
identified as a suppressor of det1. It contains a transition from G to a in the PEX2 gene, resulting
in a missense mutation of a valine to methionine substitution in front of the C-terminal RING
finger domain of the peroxisomal membrane protein encoded by PEX2. The objective of this
study is to examine the molecular details underlying the ted3 phenotype. We postulate that a
truncated PEX2 protein, which contains the RING finger domain, may be generated in the
ted3 mutant and is involved in the degradation of HY5 via the proteasome. Here, we show that the
PEX2 RING finger domain by itself localizes to the nucleus and interacts with HY5 in vivo. We
also demonstrate that overexpressing the PEX2 RING finger domain in det1 plants leads to a
partial ted3 phenotype, indicating a role of this domain in suppressing the det1 phenotype. Com pared with det1 and WT, ted3 plants have significantly decreased levels of the HY5 protein
and decreased expression of some HY5 target genes. Taken together, these observations
suggest that HY5 activity in the ted3 plants is partially compromised and that this alteration of
HY5 activity may have been responsible, at least in part, for the reversal of the det1 phenotype in
the ted3 dominant mutant.

2000/B379
Role of the AtDRP2 Family in Arabidopsis Gametophyte Development.
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Dynamins are polymerizing GTPases involved in membrane trafficking and organelle dynamics
throughout eukaryotes. In Arabidopsis there are at least six families of dynamin-related proteins
(DRPs), with functions ranging from endocytosis to chloroplast division. The AtDRP2 family has a
domain structure most similar to mammalian dynamin 1, and is putatively involved in clathrin-
mediated vesicle budding at the Golgi or plasma membrane. However, the role of AtDRP2 in
plant development has never been investigated. Here we demonstrate that AtDRP2 is essential
for both male and female gametophyte development. Female gametophytes in which both
AtDRP2A and AtDRP2B have been disrupted arrest at stage FG1, while mutant pollen aborts
around the time of the first cell division. By comparing the developmental function and subcellular
localization of AtDRP2 to that of the AtDRP1 family we can begin to unravel the roles of
endocytosis and membrane trafficking in plant development.
Dissection of the Functional Roles of Domains in Arabidopsis thaliana AGC Kinases during Pollen Tube Development and Identification of Interacting Proteins.

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Recent work has shown that AGC kinases, known to be involved in polarized animal cell growth, also play an important role in normal development and growth of pollen tubes. Normal polarity of pollen tube growth is required for successful migration of sperm to the ovule. The Arabidopsis thaliana AGC kinases, AGC1.5 and AGC1.7, are functionally redundant; activity of either will condition normal pollen tube morphology, although plants with loss-of-function alleles of both genes have reduced seed set. When pollen grains lacking AGC1.5;AGC1.7 function are germinated in vitro, they produce a pollen tube of normal width and polarity for the first 100-150 µm of growth; subsequently the tube widens and becomes twisted. This summer I investigated the critical domains of the AGC kinases by transforming constructs containing truncations and point mutations into an agc1.5;agc1.7 double mutant background. Transgenic pollen tubes and the cytoplasmic distribution of the AGC kinases were identified by fusions of the altered proteins to yellow fluorescent protein (YFP). My results show that when AGC kinases are altered to eliminate phosphorylation activity, they are unable to complement the double mutant, and that removal of the PIF domain from AGC1.5 and AGC1.7 allows only partial complementation. Finally, an alteration predicted to render the AGC kinases constitutively active permits complementation of the double mutant with no deleterious effects. I also used yeast two hybrid tests to identify proteins that interact with these AGC kinases; such proteins might be components of functional complexes or transient substrates for phosphorylation. Eight candidate proteins (selected based on literature searches for proteins that interact with similar kinases in other systems) were used as preys, and AGC1.5 and AGC1.7 were used as baits. Our results identified three proteins as likely interactors, and the relative strengths of the interactions have been quantified. Investigations are planned to determine which regions of the AGC kinases are critical for interactions with identified proteins, use of bimolecular fluorescence to demonstrate these interactions In Vivo and genome-wide screening to identify additional interactors.

Identification of a Mechanochemical Checkpoint and Negative Feedback Loop Regulating Branching Morphogenesis.

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Cleft formation is the initial step in branching morphogenesis, with clefts initiating as indentations in the basement membrane, which then progress into the interior of the epithelium. While previous studies have identified putative cleft initiator molecules, the molecular details of this process remain unclear. We hypothesized that localized actomyosin cellular contraction was required for cleft initiation, and that signaling through Rho kinase (ROCK), a downstream effector of Rho GTPase, could mediate these events. We investigated the effects of ROCK inhibition on mouse submandibular salivary gland (SMG) ex vivo organ cultures. Pharmacological inhibitors of ROCK, isoform-specific ROCK I but not ROCK II siRNAs, and inhibitors of Myosin IIb activity stalled clefts at initiation. This finding implies the existence of a mechanochemical checkpoint regulating the transition of initiated clefts into progression-competent clefts. Since fibronectin (FN) has previously been implicated in cleft formation, we investigated a function for ROCK I in FN assembly. We found that downstream of the checkpoint, clefts are rendered competent through localized assembly of FN promoted by ROCK I/myosin II. Cleft progression is primarily mediated by ROCK I/myosin II-stimulated cell proliferation with a contribution from cellular contraction.
driving further FN assembly. FN also promotes increased cell proliferation and enhances non-muscle myosin light chain 2 phosphorylation, thereby triggering a wave of contractility as the cleft progresses. We observed an increased number of initiated, but not progressing, clefts in the presence of ROCK and myosin inhibitors, thus indicating that ROCK-mediated contractility also constitutes a negative feedback loop. These results reveal that a primary point of regulation governing cleft formation is a ROCK I/myosin II-mediated biomechanical checkpoint which also initiates a negative feedback loop that locally prevents further cleft initiations at the surface of epithelial buds.

2003/B382
Engineered "Smart" Materials for Improved Cardiomyocyte Differentiation.
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Recent studies have reported the importance of extracellular matrix (ECM) elasticity in directing adult stem cells toward a specific lineage. However, such tissue-specific ECM elasticity arises from developmental changes in matrix and suggests that traditional cultures on ECM-coated glass or gels with a static set of intrinsic parameters may not be the most appropriate physical environment, especially for highly contractile cells such as cardiomyocytes. Rather, such cells should be cultured in the appropriate physical conditions that mimic tissue progression, measured here by atomic force microscopy (AFM) as changing from soft, pre-cardiac mesoderm at E1, $E_{\text{meso}} \sim 0.2 - 1$ kPa, to a contractile heart tube at E3, $E_{\text{tube}} \sim 3.4 \pm 0.5$ kPa, and finally to mature, less compliant cardiac tissue by E17, $E_{\text{cardio}} \sim 8.2 \pm 1.3$ kPa. Limited differentiation or maintenance of a contractile phenotype even on compliant materials hints that such a dynamic property may be an important differentiation regulator, and to mimic this temporal ECM change in the myocardium, we have made an engineered “smart” material. Using the AFM measurements as design parameters, the elastic properties of collagen, fibronectin and hyaluronic acid (HA) matrices were tuned to mimic in situ temporal elasticity changes via time-dependent poly(ethylene glycol) diacrylate (PEGDA) crosslinking. Stem cells were plated onto these engineered matrices, and improved cardiomyocyte differentiation was characterized by immunofluorescence after staining for proteins that mark cardiogenesis. Results from this experiment will not only have a profound impact on the field of cardiovascular engineering, but will influence the way in which many cellular regenerative therapies are conducted.

2004/B383
Oriented Matrix Promotes the Directional Development of Microvessels.
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The functional impact of the three-dimensional organization of the extracellular matrix on morphogenesis is incompletely defined because there are few tools that permit the experimental control of matrix structure in developing tissues. Here we have used a newly developed In Vitro system for producing cell-derived matrix to examine the role of matrix anisotropy in microvessel development. Human fetal lung fibroblasts were cultured for seven days and then extracted via detergent lysis. Previously, this fibroblast-derived matrix has been used as scaffold to promote formation of human endothelial cell microvessels (Soucy and Romer, Matrix Biology 28:273-283, 2009). For the present study, micropatterned fibronectin substrates were used to test the hypothesis that substrate design could be used to accomplish a three-step goal: the orientation of fibroblasts early in the matrix deposition process; subsequent predetermined orientation of the fibroblast-derived matrix; and directional control of microvessels developing in these matrix microenvironments. BioWrite substrates containing parallel lines of fibronectin with a range of line widths and pitches were tested. Protein components of the fibroblast-derived matrix were visualized by labeling with a fluorescent probe for amine groups. Microvessel networks were...
visualized by labeling the endothelial cell f-actin. Matrix and microvessel anisotropy were then examined using an autocorrelation-based approach in which directionality and the relative degree of anisotropy were computed. The resulting data showed that the fibronectin patterns oriented fibroblast attachment and growth, deposition of the three-dimensional fibroblast-derived matrix, and the spatial organization of microvessels that were formed in the fibroblast-derived matrix. These studies provide strong evidence that matrix anisotropy regulates spatial orientation during tissue morphogenesis. Further, this approach provides a robust method for the definition of the parameters and molecular determinants of this directional control, and will facilitate the incorporation of spatial orientation into tissue-engineered constructs.

2005/B384
Active Actin Cytoskeleton Remodeling Extrudes Dying Cells from the Epithelial Wall in 3D to Maintain Morphological Integrity.
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Epithelial cells form the protective lining of most internal organs such as lung, pancreas, prostate, breast and kidney. Keeping the morphological integrity of epithelia is vital to a variety of biological processes. The cells in the epithelial wall are constantly dying and being replaced. However, it is not well-understood how the dying cells are eliminated from epithelia. We report here that active actin cytoskeleton remodeling of the surrounding health cells extrudes dying cells while maintaining the epithelial integrity. We use cysts with liquid filled lumen, developed from Madin-Darby canine kidney cells cultured in matrigel, as our In Vitro model of epithelial morphogenesis. To precisely control the dying process, we developed a drug inducible system, named "suicide", which has fluorescent protein expression component built-in as the indicator. Upon the addition of chemical compound, cells expressing "suicide" will die within two hours, in a cell autonomously manner. We developed mosiac cysts using plain MDCK cells mixed with cells expressing "suicide", and adding compound to them. We found that while the "suicide" cells are dying, the surrounding healthy cells actively extrude dying cells from the epithelial wall and maintain the cyst morphology. Using a panel of pharmacological inhibitors, we surprisingly found that the extruding process is actin-dependent and myosin-independent. Currently, we are using 3D live-cell imaging to further investigate the actin cytoskeleton remodeling processes and the underlying molecular mechanism.

2006/B385
Different Matrix Metalloproteinases Play Distinct Roles during Progression of Metastatic Prostate Cancer Progression.
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Prostate cancer is the second leading cause of death and the most prevalent form of cancer in men. In patients, focal prostate tumors often contain neuroendocrine differentiation (NED), which is associated with androgen independent progression and poor prognosis. To study this disease stage, we use the transgenic cryptdin-2-directed expression of the simian virus 40 large T antigen (SV 40 T-Ag), which targets neuroendocrine lineage cells in mouse prostates and is 100% penetrant to cause neoplasias and metastasis of prostate cancer. We asked if matrix metalloproteinases (MMPs), a family of enzymes shown to remodel the microenvironment, contribute to prostate cancer development. MMP-2, -7, and -9 increase activity during prostate tumor progression in the CR2-TAg transgenic model but are expressed in different cell types: stromal, rare luminal, and macrophages, respectively. We found that CR2-TAg mice treated with AG3340/Prinomastat, a broad spectrum oral non-peptide MMP inhibitor that blocks activity of MMP-2, -9, -13, and -14, had reduced tumor burden. To determine which MMP promoted tumor growth and metastasis we took a genetic approach. Consistently, at 24 weeks, Cr2-TAg transgenic mice deficient for MMP-2 show reduced tumor burden, prolonged survival, decreased
metastasis to lungs, and decreased vessel density. Comparatively, CR2-TAg; MMP-7-/− and CR2-TAg; MMP-9-/− mice did not show reduced tumor growth or increased survival. MMP-7 contributes to the average vessel size and overall endothelial area, while MMP-9 contributes to overall tumor pathology, invasiveness, and stromal vasculature density. These results suggest distinct contributions for these proteinases to the progression of aggressive neuroendocrine prostate tumors.

**2007/B386**


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Living organisms inherently produce highly defined and well-organized tissue and organ systems capable of carrying out specific functions. However, when the function of these systems is altered via mutations their structure is often changed and may result in a number of diseased states. The identification of specific changes in tissue function and subsequent correlation to their effect on the system’s structure may lead to the development of more accurate diagnostic measures and treatment. As a first step toward this goal we have employed graph theory to develop a novel quantitative methodology that permits the identification of the structure-function relationship of a tissue’s metastatic potential. Graph theory is utilized by mathematicians and computer scientists to model relations between objects from a certain collection (graph), and has been applied to many types of networks including the World Wide Web, six degrees of separation, and protein networks. In the present study, the network to be modeled is generated using a three-dimensional In Vitro system that mimics the various stages of breast cancer along the metastatic cascade. More specifically, specific proteins of interest are fluorescently labeled at various stages along the cascade and subsequently imaged via multi-spectral confocal microscopy and used to generate cell graphs. Quantitative data is extracted from the cell graphs that provide features which represent the structural organization of the artificial tissue in multiple aspects including the spatial location of individual cells, acinar structures, and extracellular matrix organization. These features are used to map the effects of structural changes to the functional changes of the cells along the metastatic cascade. Using this approach we have successfully identified quantitative features that are specific to each stage of breast cancer (normal, pre-malignant, ductal carcinoma in situ, and invasive carcinoma). These investigations demonstrate the ability to model structure-function relationships within controlled 3D systems that holds great potential as diagnostic tools.

**2008/B387**

Nuclear Co-Repressor 2 Represses Mammary Branching Morphogenesis and Local Tumor Invasion through Epigenetic Regulation of Fibronectin.

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Mammary tissue development depends upon site-specific and coordinated alterations in cell-cell and cell-extracellular matrix (ECM) interactions that are critical for branching morphogenesis and acini differentiation. The mechanisms directing coordinated ECM remodeling and tissue development remain incompletely understood. The epigenetic modifier nuclear corepressor-2 (N-CoR2) plays an important role in regulating tissue development and mammary epithelial cell (MEC) behavior. Intriguingly, immunohistochemistry profiling of tissue microarrays demonstrated that N-CoR2 displays considerable inter-regional expression heterogeneity in the human breast epithelium. Upon further examination we observed that nuclear levels of N-CoR2 are highest in the terminal ductal lobular units (TDLU) as compared to the adjacent ductal network. Using a three dimensional organotypic assay we noted that N-CoR2 promotes acinar morphogenesis whereas its absence supports branching morphogenesis. Gene expression profiling showed that downregulation of N-CoR2 in non-malignant mammary epithelial cells (MECs) increases fibronectin (FN1) levels and reduces E-cadherin mRNA and protein expression. Importantly, reducing fibronectin expression in the N-CoR2 null MECs restored acini differentiation and
repressed branching morphogenesis, consistent with a crucial role for N-CoR2 regulation of fibronectin expression in dictating breast tissue differentiation. Chromatin immunoprecipitation further demonstrated that N-CoR2 directly binds to the FN1 promoter and represses its transcription through histone deacetylase 3 (HDAC3)-dependent chromatin remodeling. These data implicate N-CoR2/HDAC3 as an important epigenetic regulator of breast tissue morphogenesis and identify fibronectin as a key molecular target mediating this effect. Because we also observed a significant association between nuclear levels of N-CoR2 and FN1 expression in primary human breast tumors and regional lymph node metastasis we are currently exploring the relevance of this epigenetic regulatory mechanism to breast tumor invasion and metastasis. (Supported by NSC 96-2321-B-038-004 to KKT and DOD BCRP W81XWH-05-1-330 and NIH CA078731 to VMW).

2009/B388
The Role of β1-Integrin in Mammary Gland Ductal Morphogenesis.
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Interaction between mammary epithelial cells (MEC) and the extracellular matrix is essential for the development of mammary gland tissue. The formation of mammary ducts requires cell-matrix interactions via β1-integrins(1). Moreover, when β1-integrin is deleted from the MEC during pregnancy the glands subsequently exhibit disorganised alveoli(2,3). MEC become detached from the basement membrane and protrude into the luminal cavity. This resembles the phenotype in very early breast cancers including atypical ductal hyperplasia and some ductal carcinoma in situ. The aims of this project are: to determine if ductal formation in the mammary gland is driven by luminal cells, basal/myoepithelial cells or a combination of both to examine the effects of β1-integrin deletion in this branching process, especially in specific cell types to investigate the role of mammary stem cells in ductal morphogenesis We are using an In Vitro culture model that mimics mammary gland development in vivo. Primary MEC from virgin mice were embedded into Matrigel and cultured with FGF2, which induced the organoids to form protrusions that resembled ductal branching. To identify the cell types involved in this process, we are currently separating MEC into luminal and basal/myoepithelial populations by flow cytometry(4) and culturing them in the organotypic 3D system. Cellular outgrowth is being tracked using lentiviral infection with RFP-H2B, combined with 4D spinning disc confocal imaging(5). Using this system we will carry out cell type specific deletion of β1-integrin in primary MEC isolated from β1-integrinfx/fx; CreERTam mice. The gene will be knocked out at time of plating and after the branching structures have been established. We are testing the hypothesis that in the absence of β1-integrin, the cells will lose their polarity(6) and become disorganized, proliferation will be impaired, and ductal branching will be disrupted. (1) Klinowska et al., 1999 (2) Naylor et al., 2005 (3) Li et al., 2005 (4) Sleeman et al., 2005 (5) Ewald et al., 2008 (6) Akhtar et al., manuscript in preparation

2010/B389
3D Morphogenesis and Collective Migration of Epithelial Cells Observed on a Soft Substrate Containing Laminin.
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Epithelial morphogenesis such as cyst and tubule formation in 3D environment is essential events in diverse physiological processes, including embryonic development and angiogenesis. However, the dynamical behavior of epithelial cells during lumen formation and the underlying mechanism are less well understood. Our previous studies demonstrated that collective cell movement occurred during the lumen formation when an epithelial sheet (MDCK cells) on a collagen gel was overlaid with another collagen gel. A turnup of the periphery of the epithelial sheet appeared within several hours after the gel overlay, and then the collective cell movement continued until the cells completed the lumen formation. In the present study, we cultured MDCK cells on a soft substrate containing laminin and performed time-lapse observation by phase
contrast microscopy. We found that the cells tugged at the peripheral matrix and remodeled the gel surface morphology, resulting in the lumen formation above the gel surface. The appearance is like a tulip hat or a croissant. Immunofluorescence studies showed that the epithelial cells secreted laminin matrix. The lumen formation was not observed on a hard substrate such as a coverslip coated with laminin. These results indicate that not only laminin but also a mechanosensing process would be involved in the 3D morphogenesis on a soft substrate.

2011/B390
Defining the Role of ECM in Wound Healing Mechanisms.
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Two general modes of collective migration have been identified in embryogenesis and wound healing. The first, often termed ‘lamellipodia crawling’, is characterized by the migration of separated or connected cells from the wound edge into the wound area. The second depends on the collective development of peripheral actomyosin bundles in the cells at the leading edge that close the wound through a ‘purse-string’ contraction. It has been suggested that both modes depend on the presence of ECM and wound geometry, but the mechanism of how one mode is selected over the other is still unexplored. In this work, we attempt to study the interplay between ECM, wound geometry and the resulting healing mechanism. Using soft lithography, we fabricated polydimethylsiloxane (PDMS) stamps bearing micron-sized posts of different shapes and sizes. Stamps were inverted over glass coverslips which were pre-coated with artificial extracellular matrix proteins (aECM) containing either the RGD or the scrambled RDG cell-binding domain. Cells were then grown to confluence underneath the stamps, after which the stamps were removed to reveal wound areas of controlled geometry and aECM composition. The process of healing was subsequently observed using time-lapse microscopy. Spatial patterns of mode selection were observed during wound closure. The lamellipodia crawling mode was found to interleave with the purse-string mode with a spacing that depended on wound size, wound shape and the RGD density in the wound area. In particular, the lamellipodia crawling mode dominated on wound areas containing high RGD density whereas wound areas with only RDG facilitated the selection of purse-string mode. These observations led us to propose a ‘lateral inhibition’ model where both lamellipodia crawling and purse-string modes competed with one another. Using this model allowed us to make predictions on how the kinetics and pattern formation of lamellipodia crawling and purse-string modes contributed to wound closure, and ultimately better define the role of ECM in wound healing.

2012/B391
Tenascin C Regulates Recruitment of Smooth Muscle Cells during Coronary Arterial Development.
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During heart development, coronary vascular system, which is required to nutrient supply for thick compact layer of cardiac wall, is established from proepicardial mesenchyme via vasculogenesis followed by angiogenesis. Tenascin C (TNC) is an extracellular glycoprotein and is thought to be involved in tissue remodeling during organogenesis and regeneration in the pathologic legions. Using quail embryonic hearts, we investigated spatiotemporal expression patterns of TNC during the establishment of proximal region of coronary arteries connecting with aortic sinuses. Immunohistochemistry showed that at ED5 (prior to the formation of coronary stem), TNC was deposited circumferentially in the tunica media of ascending aorta and pulmonary trunk. at this time, anti-smooth muscle actin (SMA) immunoreactivity was weak in the proximal region of ascending aorta and pulmonary trunk. at ED6, endothelial strands, primordial coronary stems, invaded into the aortic wall, in which TNC was deposited. In proximal pulmonary trunk, deposition
of TNC was sparse but the expression of SMA was apparent. at ED8, when a single orifice of coronary stem was completed and SMA-positive cells were recruited, TNC was extensively deposited surrounding the coronary arterial wall. Quail-chick chimera experiment showed that proepicardial organ gave rise to SMA-positive cells making up coronary arterial tunica media and expressing TNC. When nascent proepicardium was cultured on dish coated with purified TNC, cells differentiated into smooth muscle cell phenotype expressing SMA. Epicardial cells cultured without TNC showed a typical cobblestone appearance with expressing endothelial marker OH1. These results revealed that TNC was expressed where coronary arterial stems penetrated and the expression of TNC preceded the expression of SMA, suggesting TNC have a role in recruitment of smooth muscle cells during coronary artery development.

2013/B392  
Mixture of Syndecan- and Integrin-Binding Peptides Accelerates Cell Adhesion By Their Synergetic Cellular Interactions.  
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AG73 (RKRLQVQLSIRT) and EF1 (DYATLQEGRLHFMDLG), previously identified from the mouse laminin α1 chain LG4 module, specifically bind to syndecan and α2β1 integrin, respectively. AG73 shows cell attachment with membrane ruffling and EF1 promotes cell attachment and spreading with well-organized actin. Here, we examined cell attachment and cell spreading using a mixture of AG73 and EF1 (AG73/EF1) to evaluate their synergetic effect through the syndecan- and integrin-binding mechanism. First, we examined the time course analysis of cell attachment on AG73, EF1, and AG73/EF1 (molar ratio=1:1). The cells on AG73 attached fast and the number of attached cells reached a plateau at 40 min. In contrast, the cells on EF1 attached slowly and the number of attached cells reached a plateau at 90 min. The mixed peptide AG73/EF1 showed the fastest cell attachment and the amount of attached cells reached a plateau at 20 min. We also analyzed cell spreading by determining cell area on AG73, EF1, and AG73/EF1. Time course analysis indicated that the cell area on AG73/EF1 expanded faster than that on AG73 and EF1. These results suggest that synergetic cooperation of syndecan and integrin accelerate the cell attachment and cell spreading. Next, we evaluated the Tyr397 phosphorylation of FAK on AG73, EF1, and AG73/EF1 to analyze the cellular signaling mediated by two different cell surface receptors. AG73 promoted weak Tyr397 phosphorylation and EF1 promoted strong Tyr397 phosphorylation as reported previously. AG73/EF1 promoted the strongest Tyr397 phosphorylation indicating that the synergetic cooperation enhanced the intracellular signaling. These results suggest that a peptide mixture of AG73 and EF1 accelerate cell adhesion processes mediated by synergetic receptor interactions of syndecan and α2β1 integrin. We conclude that the mixed peptide approach has potential for use as a multifunctional biomaterial and that this may be a promising method for analyzing mechanisms of multi-receptor dependent cellular functions.

2014/B393  
Integrins and a Thrombospondin from the Anthozoan Nematostella Vectensis.  
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Thrombospondins (TSPs) are a family of extracellular matrix proteins that influence cell behavior through multiple receptors, including integrins. Unlike the integrin ligands fibronectin and tenasin, which are only found in the Phylum Chordata, TSPs have been identified in both deuterostome and protostome lineages. Here we report the cloning and predicted protein sequence of a TSP from the sea anemone Nematostella vectensis. The cDNA sequence encodes a protein with a TSP-type N-terminal domain, 4 EGF-like domains, 13 type 3 repeats and a C-terminal L-lectin-like domain. This is the same domain organization as members of the mammalian TSP subgroup B (TSP-3, TSP-4 and COMP/TSP-5); however, the Nematostella TSP has only a short coiled-coil and lacks the paired cysteine residues that also contribute to
pentamerization of vertebrate subgroup B TSPs. The Nematostella TSP contains the sequence DGDGRGDACDD, which has been shown by others to be an integrin recognition site. Whole mount in situ hybridization demonstrated widespread expression of the TSP gene in juvenile polyps, but not in planula larvae. Following polyp transection the TSP gene is expressed in the regenerating blastema. Genes encoding three other TSP-like proteins were also identified in the Nematostella genome, and their transcription was confirmed by RT-PCR. RT-PCR was also used to identify the transcription of one alpha and four beta integrin subunit genes in juvenile polyps. The presence of a transcript encoding a TSP with an RGD motif in Nematostella, as well as the expression of integrin genes, suggests the possibility that RGD-dependent interactions between TSPs and integrins could provide a fundamental mechanism for extracellular matrix-mediated signaling.

2015/B394
Dystroglycan Controls Stat5 Activity and Growth Hormone Signaling.
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Defects of the basement membrane receptor dystroglycan arising in muscle and nervous tissues produce muscular dystrophies and congenital brain defects. Similar defects of dystroglycan arise in the progression of many carcinomas and may contribute to cancer progression. To assess dystroglycan functions within an epithelial lineage in vivo, we generated the conditional knockout of dystroglycan in the mouse mammary gland. Glands lacking dystroglycan displayed an inhibition of epithelial outgrowth that coincided with a failure of lactation. This striking phenotype was associated with a marked deficiency in activation of the signal transducer and activator of transcription 5 (STAT5), a critical regulator of mammary gland development and function. Importantly, loss of dystroglycan function perturbed STAT5 activation in response to both prolactin and growth hormone signaling, as assayed by the induction of phospho-STAT5 and STAT5-dependent gene expression (milk proteins and insulin-like growth factor-1) in cultured mammary epithelial cells. These results reveal a selective and potent role for basement membranes and dystroglycan in the modulation of STAT5 activity in vivo, with implications for multiple STAT5-dependent developmental pathways, and for the manifestations of muscular dystrophies and cancers wherein dystroglycan function is compromised.

2016/B395
Periostin Expression in the Human Periodontal Ligament: Role of Focal Adhesion Kinase and Transforming Growth Factor Beta 1.
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The periodontal ligament is an essential tooth-supporting structure, mediating the dispersion of applied mechanical forces resulting from mastication. Composed primarily of type I collagen, little is known about other matrix components that form an integral part of the ligament. Recently identified as a key component of the murine periodontal ligament, the matricellular protein periostin is thought to regulate collagenous tissue response to mechanical strain. The aim of this study was to assess if periostin is expressed in the human periodontal ligament, and to assess potential mechanisms of regulation. Using immunohistochemistry, periostin was identified in the periodontal ligament, terminating at the dentogingival junction. Immunoreactivity was significantly reduced in teeth from which occlusal loading was absent. When cultured in 3D type I collagen gels in vitro, periodontal ligament fibroblasts elevated periostin mRNA levels in response to Transforming growth factor beta 1 (TGFβ1), which corresponded with increased collagen gel contraction. Addition of LY364947 (ALK-5 inhibitor) or PP2 (FAK/Src inhibitor) reduced collagen gel contraction and periostin mRNA levels in comparison with controls. Addition of PP2
attenuated nuclear translocation of twist-1, a known periostin transcription factor. Stimulation of fibroblasts with TGFβ1 in the presence of PP2 confirmed that FAK/Src signaling in part mediates the cell response to TGFβ1. Confirmation that FAK is required for periostin expression was performed using FAK null fibroblasts, which contained no detectable periostin mRNA. In conclusion, periostin is expressed in the human periodontal ligament in situ, and its expression is mediated by both TGFβ1 and FAK/Src signaling.

**Integrins II (2017 – 2030)**

2017/B396

**Integrin B1 Expression by Fibroblasts Is Required for Tissue Repair In Vivo.**

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In tissue repair, fibroblasts migrate into the wound to produce and remodel extracellular matrix (ECM). The role of specific integrins in this process is poorly understood. Here, we show that mice containing a fibroblast-specific deletion of integrin β1 exhibit delayed cutaneous wound closure, and reduced production of collagen and α-smooth muscle actin (α-SMA)-expressing myofibroblasts at the wound site. Integrin β1-deficient fibroblasts showed reduced expression of type I collagen and connective tissue growth factor and failure to differentiate into α-SMA-expressing myofibroblasts. Integrin β1-deficient fibroblasts also showed α-SMA stress fiber formation, and reduced abilities to adhere to, spread on and contract ECM. Integrin β1-deficient fibroblasts showed reduced generation of reactive oxygen species (ROS) and rac1 activity; overexpressing rac1 rescued the ability of integrin β1-deficient fibroblasts to generate ROS, and hydrogen peroxide partially alleviated the phenotype of cultured integrin β1-deficient fibroblasts. Moreover, integrinβ1-deficient cells appear to possess defects in TGFβ activation; addition of TGFβ rescues the In Vitro phenotype of these cells. Thus integrin β1 is essential for normal wound healing, acting at least in part through impaired TGFβ and rac1/ROS-signaling.

2018/B397

**DMSA-Functionalized Magnetic Nanoparticles Induce Transendothelial Migration of Monocytes in the Mice’s Lung By B2 Integrin-Dependent Pathway.**

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Magnetic nanoparticles surface-functionalized with meso-2,3-dimercaptosuccinic acid (MNPs-DMSA) presents preferential distribution for the lung. This target specificity offers a property useful to treat lung diseases. However, the presence of MNPs-DMSA in the lung leads to transendothelial migration of leukocytes. In the present study, the kinetic of migration activity, leukocyte subpopulation, macrophage up-take, MNPs-DMSA citotoxicity on macrophage in vitro, and cell adhesion molecule pathway behind this process were investigated. We found that MNPs-DMSA caused a time-dependent increase in the number of leukocytes in bronchoalveolar lavage fluid within the first 12h (P<.05). Thereafter, the number of leukocytes decreased (P<.05), returning to initial values at 72h. The cytologic and phenotypic analysis of bronchoalveolar lavage cells revealed that macrophages were the predominant cellular component in all analyzed periods (0, 4, 12, 24, 48 and 72h). Analysis ultrastructural of cells obtained from bronchoalveolar fluid after MNPs-DMSA administration (4 and 12h) revealed the presence of nanoparticles inside of numerous macrophages. MNPs-DMSA at the concentrations tested showed no toxic effects to alveolar macrophages, as evidenced by MTT* assay. In contrast to β2-integrin LFA-1, β1-integrin VLA-4 and immunoglobulin VCAM-1, MNPs-DMSA induced the up-regulation of E-, L- and P-selectin and β2-integrin Mac-1 in the lung (P<.05). Finally, the β2-integrin-dependent pathway elicited by MNPs-DMSA was demonstrated by use of knockout mice. Our results point out towards a strategy to improve functional properties of MNPs-DMSA during biomedical applications in the lung, for instance by its use combined with β2-integrin-inhibitors. *3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
2019/B398

Integrin Alpha4 Expression Is Involved in Trophoblast Invasion during Early Implantation.

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Implantation is initiated by an attachment of the trophectoderm to the maternal endometrial epithelium. The processing is a complex, dynamic and tightly controlled for embryo development. Especially, these interactions are regulated by the coordinated interplay of various growth factors, cytokines, hormones, and cell adhesion molecules depend on microenvironment such as hypoxia. Integrins are adhesion receptors that mediate both cell-extracellular matrix and cell-cell interactions. However, roles of integrin-a4 in trophoblast during early implantation have not been explored. The objective of this study was to analyze the expression of integrin-a4 in trophoblast exposed to hypoxic condition and to demonstrate the correlation between trophoblast invasion and integrin-a4 expression. The collected trophoblast cells after 1% hypoxia treatment according to time courses were examined by RT-PCR, western blot, zymography and cell invasions were estimated. The expression of integrin-a4 in early time point were significantly down-regulated than late time point. The expression of human matrix metalloproteinase (MMP)-9 in early time point was increased in trophoblast exposed to hypoxic condition. In addition to, invasiveness of trophoblast cultured in 1% hypoxia was significantly increased than normoxia (p<0.05). Taken together, these results suggest that alteration of integrin-a4 expression by hypoxia has a role in regulating the trophoblast invasion during early implantation.

2020/B399

Activity of Kv1.3 Has No Functional Importance for B1 Integrin Mediated Cell Adhesion in T Cells.

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Adhesion of T lymphocytes to extracellular matrix (ECM) components is an important part of immune surveillance and of a functional immune system. Activation of human T cells by antigen stimulation leads to activation of integrins pivotal for cell adhesion. Functional association between β1 integrins and the voltage-gated potassium channel Kv1.3 has previously been described in human T cells (Levite et al. J. Exp. Med. 2000 Vol. 191 pp. 1167). Elevated extracellular K+ and hence opening of Kv1.3 channels was shown to be sufficient for induction of β1 mediated adhesion. This adhesion could furthermore be inhibited by the Kv1.3 specific blocker margatoxin. We investigated if such an association exists by examining the significance of Kv1.3 channels on β1 mediated adhesion to the ECM component fibronectin. To assess this cell adhesion we used impedance measurements (ACEA Biosciences) and either human T cells or Jurkat cells. Our results show that β1 integrins are responsible for the majority of adhesion to fibronectin and that elevated extracellular K+ has no stimulatory effect on this adhesion. In addition we were unable to see any inhibition of adhesion when using margatoxin to block Kv1.3 channels. Margatoxin was also unable to inhibit the increased adhesion to fibronectin seen when T cells were stimulated with an anti-CD3 antibody. This increased adhesion was also shown to be β1 integrin mediated. Taken together our results question the existence of a functional association between β1 integrins and Kv1.3 channels in human T lymphocytes.

2021/B400

β1d Integrin Plays a Role in Anisotropic Stretch-Induced Hypertrophy.

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In healthy myocardium, cardiomyocytes hypertrophy in response to increased load, and expression of atrial natriuretic factor (ANF) rises. In cultured cardiomyocytes, previous work has shown that transverse stretch increased hypertrophic signals more than longitudinal stretch. Due
to its location in the costamere and known association with cytoplasmic signaling molecules, we hypothesized that integrins would play an important role in the directional sensitivity of mechanotransduction in cardiomyocytes. Neonatal rat ventricular myocytes were cultured in an aligned pattern on anisotropic stretching devices. The cells were then infected with an adenovirus to disrupt native integrin function (Tac β1D) or a control virus (Tac α5), and stretched predominantly along their longitudinal or transverse axes for 24 hours. Real time quantitative PCR was performed to analyze changes in ANF gene expression, as a marker of the myocyte hypertrophic response. ANF expression levels in the stretched cells were normalized to unstretched cells. Control (Tac α5 integrin infected) cells displayed ANF expression in transversely stretched cells that was significantly higher than in longitudinally stretched cells. ANF levels were lower in stretched myocytes with disrupted integrin function (Tac β1D infected cells) compared to the control cells. Most importantly, when integrin function was disturbed, ANF expression in transversely stretched cells dropped below levels present in longitudinally stretched cells. These results suggest that β1D integrin, the dominant β integrin in post-natal cardiac myocytes, plays a role in anisotropic stretch-induced hypertrophy. The spatial distribution of β1D integrin in the costamere, which is located circumferentially around the cell, may be responsible for this directional sensitivity to mechanical stimuli.

2022/B401
Neuronal Thy-1 Induces Cell Adhesion in Astrocytes via Biphasic Activation of RhoA.

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Thy-1 induces astrocyte adhesion by engaging both αVβ3 integrin and syndecan-4 present in astrocyte membranes in a PKCα and RhoA/ROCK-dependent manner. RhoA is reportedly activated downstream of PKCα; however, whether the activation of PKCα and RhoA occur downstream of integrin, syndecan-4, or both receptors is currently unknown. Thus, the sequence of events triggered by integrin and/or syndecan-4 engagement was studied. To this end, DI TNC1 astrocytes were stimulated with the wild type Thy-1-Fc or mutants of Thy-1-Fc lacking integrin- or syndecan-binding sites. Using these recombinant proteins in the presence or absence of pharmacological inhibitors, we evaluated morphological changes by immunofluorescence analysis, RhoA activation by pull-down assays and PKCα activation by immunoblotting with anti-phospho-specific antibodies at different time points. Analysis of RhoA and PKCα activation revealed two peaks of activity, one before 10min and a second after 20min of Thy-1-stimulation. Focal adhesion formation was observed at 10 and 30min of stimulation with the wild type Thy-1-Fc, but not with the mutated Thy-1-Fc proteins. Thy-1-Fc effect was attenuated by PP2 (Src inhibitor) and U73122 (PLCγ inhibitor). The first peak of RhoA activation was also blocked by PP2 and U73122. These results suggest that Thy-1 triggers sequential activation of two different pathways involving Src, PLCγ, PKCα, and RhoA and that both integrin- and syndecan-binding sites are important for this response. Acknowledgements: FONDECYT 1070699 (LL); CONICYT 24080094 (AV); FIRCA 1RO3 TW007810-01A1 (KB-LL); FONDECYT 1090071 and FONDAP 15010006 (AFGQ).

2023/B402
Identification of the Laminin Alpha5 Binding Site with in Lutheran B-Cam Using a Function-Blocking Antibody.

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Lutheran blood group glycoprotein (Lu), an Ig superfamily transmembrane receptor, is also known as basal cell adhesion molecule (B-CAM). Lu B-CAM is expressed on the surface of red blood cells, on endothelial cells of capillaries, and on diverse epithelial cells. Lu B-CAM is a specific receptor for laminin alpha5, a component of basement membranes. Previous reports have shown that Lu B-CAM binding to laminin alpha5 contributes to sickle cell vaso-occlusion. However, it
has not yet been possible to regulate the adhesion of sickled red blood cells to laminin alpha5. By studying several monoclonal antibodies that recognize Lu B-CAM, we discovered a function-blocking antibody that inhibits the binding of Lu B-CAM to laminin alpha5. Here we identified the region on Lu B-CAM that is recognized by this antibody. Lu B-CAM is a glycoprotein modified by N-linked glycosylation. To determine whether the antibody recognizes sugar chain or amino acids, a recombinant protein containing the Lu B-CAM extracellular domain was denatured under reducing conditions and deglycosylated with glycopeptidase F. The deglycosylated protein was detectable by immunoblotting, indicating that the antibody recognizes the core protein. The extracellular region of Lu B-CAM contains two variable and three constant Ig-like domains, V-V-C2-C2-C2. To narrow the region recognized by the antibody, we produced a series of truncated proteins with sequentially deleted Ig-like domains. These proteins were fused with human IgG1 Fc for purification. The antibody was diluted with PBS containing the mutant recombinant proteins and used for ELISA. Although the antibody recognized V-V-C2-C2-Fc, V-V-C2-Fc, and V-V-Fc, V-Fc lost immunoreactivity. These results show that the antibody epitope is localized in the second variable Ig-like domain. Precise epitope mapping may be useful for developing drugs to inhibit the vaso-occlusion common in sickle cell disease.

2024/B403
Beta1 Integrin Inactivation and Disruption of Focal Adhesions by the Distintegrin Contortrostatin.
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Integrins mediate signaling in a multitude of cellular pathways and their activation status plays an important role in their ability to interact with the extracellular matrix. Disintegrins have long been known to act as integrin antagonists with the ability to disrupt adhesion based integrin functions through their direct binding. Based on evidence accumulated while investigating the action of contortrostatin (CN), the homodimeric disintegrin isolated from southern copperhead venom, on actin cytoskeleton disruption, we hypothesized that disintegrins can act on signaling pathways and affect the activation state of integrins. Here we demonstrate that talin, a key molecule in integrin activation, can be directly affected by CN ligation of beta1 integrins. Talin binding to the cytoplasmic domain of integrins is the final step in integrin activation and through a series of co-immunoprecipitations and western blotting we discovered, following a 30-min CN treatment of MDA-MB 231 cells, that there is a CN-mediated rapid displacement of talin from the cytoplasmic domain of beta1 integrins. We also observed a decrease in important binding interactions with molecules such as Rap1 and Rap1-interacting adaptor molecule (RIAM) possibly halting the recruitment of talin to the plasma membrane. Further, using HUVEC cells, we found that after 18-hour exposure to CN we observed a decrease in calpain-II cleavage of talin into its head and rod domains inferring that talin was being left in its autoinhibited conformation. These events combine to produce a global inactivation signal of beta1 integrins as indicated by flow cytometry and a decrease in the ability of the cell to produce talin based focal adhesions as shown by confocal microscopy.

2025/B404
Integrin-Linked Kinase (ILK) Is Required for Transforming Growth Factor-β Induction of Fibroblast Differentiation.
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The skin is an organ with exceptional regenerative capacity. Following cutaneous injury, the epidermal keratinocytes and dermal fibroblasts of the skin are activated to reepithelialize the wound. Dermal fibroblasts acquire a migratory and proliferative phenotype, differentiating into myofibroblasts. The latter, which are critical for wound contraction and closure, arise from fibroblast responses to a variety of cytokines and growth factors produced at the wound site, including transforming growth factor-β (TGF-β). Indeed, TGF-β is a well-established promoter of myofibroblast differentiation, and induces expression of α-smooth muscle actin (SMA), which is
key for wound contraction. We have investigated the role of integrin-linked kinase (ILK) in differentiation of primary cultured murine dermal fibroblasts into myofibroblasts in response to TGF-β. Inactivation of the Ilk gene results in impaired differentiation into myofibroblasts. Specifically, ILK-null fibroblasts exhibit defects in proliferation and viability, as well as chemotactic migration towards serum or TGF-β. Further, ILK-null fibroblast populated collagen lattices show pronounced impairment in contraction. These alterations are associated with defective activation of mitogen-activated protein kinases, reduced production of α-SMA, and alterations in focal adhesion assembly. Together, these observations indicate that ILK is essential for the ability of dermal fibroblasts to differentiate into myofibroblasts in response to injury-induced signals, and suggest that ILK is a key component for skin regeneration. Supported by the Canadian Institutes of Health Research and the National Sciences and Engineering Research Council of Canada.

2026/B405
Talin1 and Talin2 during Cardiac Development and Hypertrophy.
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Cardiac costameres are sub-sarcolemal structures that align with the Z-disc in myofibrils. They function in muscle-extracellular matrix adhesion and in mechanotransduction. Integrins are important costameric proteins. Their function and signaling are critical for normal cardiac development and response to stress. Talin is an essential integrin-actin linker and an important integrin activator. Vertebrates contain two talin genes tln1 and tln2 that are reportedly highly expressed in heart. The aim of this study was to evaluate tln1 and tln2 expression in mouse embryos and in adult basal and stressed heart. Microscopic analyses using β-gal gene-trap mice and isoform-specific antibodies showed that tln2 was expressed strongly in heart by embryonic day (E)9 and throughout cardiac development. within the heart, tln2 was detected in myocytes, but not in endothelial cells (EC). Tln1 was also detected in E9-12 heart but was expressed in both myocytes and ECs. In adult heart, tln2 was detected mainly in cardiomyocytes and specifically in costameres, whereas tln1 was predominantly expressed in non-myocyte cells. Pressure loading of the murine heart was performed to induce cardiac hypertrophy. Real-time RT-PCR of transcripts showed increased expression of tln1 but not tln2 in hypertrophic hearts and myocytes (cardiac tissue - tln1, 1.38 ± 0.16; tln2, 0.91 ± 0.09 fold change vs. sham, *p ≤ 0.05, n=4; cardiac myocytes - tln1, 1.36 ± 0.18 fold change vs. sham, *p ≤ 0.05, n=3). Immunomicroscopy showed tln1 localised to costameres in pressure-loaded heart, while none was detected there in the basal state. These results indicate that tln2 is preferentially expressed in cardiac muscle, while tln1 has a more ubiquitous distribution pattern. Expression of tln1 in embryonic heart and its increased expression in hypertrophic cardiac myocytes suggest that tln1 is important in normal heart development and in the adaptive response of the adult myocardium to stress.

2027/B406
The A2 Integrin Ectodomain Suppresses Pancreatic Adenocarcinoma Cell Invasion via Regulation of Kallikrein-Related Peptidases.
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Previous reports demonstrated the central role of the α2-integrin in mediating the interaction of pancreatic ductal adenocarcinoma (PDAC) cells with the collagens. We recently reported that poorly-differentiated PDAC cells demonstrate loss of reliance on α2-integrin (α2) for adhesion and migration on collagenI, or complete loss of α2 integrin altogether. Further, well-differentiated PDAC lines exhibit reduced In Vitro invasion compared to poorly-differentiated lines, and α2-blockade suppresses invasion of well-differentiated lines exclusively. Based on these data and the demonstrated role of α2 in maintaining tissue architecture in other organs, we hypothesized that α2 may actually suppress the malignant phenotype in PDAC. Accordingly, stable ectopic expression of α2 in endogenously α2-negative, poorly-differentiated MIAPaCa2 cells promoted
α2-dependent adhesion and migration on collagen I, and also retarded in Vitro invasion. MIAPaCa2/α2 cells maintained on CI were more invasion-retarded than MIAPaCa2/α2 cells maintained in standard tissue culture, and demonstrated higher αβ1-integrin expression that was dependent on maintained culture on collagen I. Continued growth on collagen also exacerbated the anti-invasive effect over time, suggesting the need for an accumulation of gene expression changes. Affymetrix gene expression profiling revealed that kallikrein-related peptidases (KLK)-5,6,7 were specifically upregulated by α2. Accordingly, well-differentiated PDAC lines express KLK-5,6,7 exclusively, and KLK blockade increased invasion as well as collagen I migration in KLK-positive cell lines. Importantly, an α2 cytoplasmic deletion mutant promoted KLK-5,6,7 expression exclusively and retarded invasion, while an α9α2 chimera retarded invasion less efficiently, and did not impact KLK-5,6,7 expression. These data demonstrate for the first time that the α2-ectodomain and KLK's coordinately regulate a less invasive phenotype in PDAC cells.

2028/B407
Deletion of Av Integrins in Epithelium Promotes Tumor Progression.
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The alpha v family of integrins has been implicated in many aspects of tumor progression, and increased expression of various family members has been associated with aggressive tumor growth and metastasis. A conditional knockout of the αv integrin subunit in mammary epithelium showed no obvious abnormalities in mammary development. However, when crossed with the mammary-specific MMTV- Polyoma middle T antigen transgenic mice, the spontaneous carcinoma developed with a more aggressive phenotype. The initial incidence of tumors was significantly accelerated and the deletion of αv integrin resulted in, on average, twice the total tumor burden than in control transgenics. Lung metastases were rarely observed in control tumor-bearing animals compared with nearly ubiquitous and numerous metastases found in animals lacking αv in primary tumors. Mammary epithelial cells are maintained through a number of homeostatic mechanisms, including suppression of cell proliferation by the pleiotrophic cytokine TGF-β. It is also known that αv integrins play a major role in the In Vivo activation of latent TGF-β and this activation is a major control point for this potent growth regulator. Therefore, our results are consistent with failure of αv-deficient tumor cells to activate TGF-β in the developing tumor microenvironment and are supported by data showing the loss of phospho-Smad2/3 in αv-deficient tumors. These data provide firm evidence for a role of αv integrins in local activation of TGF-β and suppression of mammary epithelial proliferation and show that early loss of αv expression accelerates tumorigenesis.

2029/B408
Regulation of Integrin A4β1 Activity By Pi3kinase Alpha Promotes Lymphangiogenesis and Tumor Metastasis.
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Lymphangiogenesis, the development of new lymphatic vessels, promotes tumor metastasis. Identification of mechanisms that drive lymphangiogenesis may help to identify new therapies for cancer and for other lymphatic disorders such as lymphedema and lymphatic malformations. We found that integrin α4β1 promotes lymphangiogenesis in primary tumors and in tumor-draining lymph nodes. This integrin is not expressed on normal lymphatic vessels but is strongly induced on proliferative lymphatic vessels in response to VEGF-A, VEGF-C and bFGF and on lymphatic vessels in tumors and tumor draining lymph nodes. Antagonists of integrin α4β1 and mutations that suppress integrin α4β1 expression (Tie2Cre α4fl/fl) or activation/signaling (α4Y991A)
suppress growth factor- and tumor-induced lymphangiogenesis. Our studies show that integrin α4 interaction with paxillin is required for integrin α4 mediated lymphatic endothelial cell motility and lymphangiogenesis. Paxillin is a scaffolding protein that binds to many signaling molecules that are crucial in promoting migration. Importantly, paxillin directly associates with integrin α4β1 in lymphatic endothelial cells and this interaction is disrupted by a Y991A mutation in the α4 cytoplasmic tail. In myeloid cells, PI3kinase γ promotes paxillin-α4 interactions and inhibitors of PI3kinase γ prevent myeloid cell integrin activation, adhesion and migration. We found that inhibitors of PI3kinase α but not PI3kinase γ block VEGF-C stimulated lymphatic endothelial cell migration and lymphangiogenesis, suggesting that PI3kinase α may promote integrin α4 activation in lymphatic endothelial cells. We are continuing to determine that mechanism by which VEGF-C promotes lymphangiogenesis by regulating integrin α4β1 activity. Our studies show that PI3kinase α promotes lymphangiogenesis by regulating integrin α4β1 mediated cell migration and suggest that PI3kinase α may regulate integrin α4 paxillin interactions during this process. These studies suggest that inhibitors of integrin α4, PI3kinase α and other signaling intermediates may be useful in suppressing tumor lymphangiogenesis and tumor metastasis.

2030/B409
Integrin Profile and Adhesion Patterns of MDR1+Oct4+ Prostate Cancer Progenitor Cells.
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Background- Recently, we identified MDR1+Oct4+ prostate cancer stem cells (PCSCs) in human prostate cancer tissues. The integrin profile of such progenitors is currently unknown and may affect their In Vivo homing characteristics. In this study, we determined the integrin profile of prostate cancer progenitors and adhesion to extracellular matrix (ECM) proteins In Vitro and in vivo. Methods and Results—PCSCs were isolated from prostate cancer tissue subjects and expression of surface integrins and adhesion to several ECM proteins were determined. PCSCs had high expression of β1 integrin, moderate expression of α1, low levels of αvβ3, and did not express αvβ5, β2, α2β1, or α4β1 integrins. In contrast, peripheral blood derived MDR1+Oct4+ cells from prostate cancer patients had high expression of α2β1, αvβ3, αvβ5, β1, and α1 and minimal expression of α4β1. Moreover, PCSCs showed increased adherence to fibronectin and collagen type I compared with vitronectin, consistent with their integrin profile, and demonstrated a similar degree of In Vivo attachment to fibronectin-coated mesh. Conclusion—These data for the first time show a spectrum of integrin expression on prostate cancer progenitors and suggest the potential importance of integrins in mediating adherence of PCSCs to specific ECM both In Vitro and in vivo.

2031/B410
Cadherin Dynamics in Adherens Junctions.
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In this work we studied the molecular dynamics of cadherin in individual adherens junctions using E-cadherin tagged by the photoconvertible protein Dendra2 (Ec-Dendra). Green-to-red photoactivation of Ec-Dendra in the individual adherens junction of A-431 and CHO cells allowed us to trace the fate of the junctional cadherin and the rate of its exchange for a nonactivated green form. Consistent with our previous biochemical data, these live-cell imaging experiments showed that adherens junctions recruit only a small fraction (about 5%) of the total E-cadherin pool. Furthermore, the residence time of an individual cadherin molecule in the junction is just a few minutes. ATP depletion completely blocked the exit of cadherin molecules from the junctions, leading to the immediate junctional entrapping of nearly all available Ec-Dendra. These data show that active processes continuously remove cadherin from the adherens junctions. This
process is not a clathrin-dependent endocytosis. To assess the role of catenins in these dynamics, we studied a cadherin mutant lacking both p120- and beta-catenin-binding sites and stabilized on the cell surface by inactivation of two endocytic elements, K738 and dileucine motif. We found that this mutant has very low rate of junctional turnover. Interestingly, catenins are not required for cadherin recruitment into the junctions since this mutant clusters with faster kinetics compared with the intact cadherin. These data show that an individual adherens junction is a continuously renewing structure.

2032/B411
Plakophilin-1 Prevents Desmosome Disassembly by Pemphigus Vulgaris IgG.
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Desmosomes are structurally complex intercellular junctions that mediate strong cell-cell adhesion between neighboring epithelial cells. In the skin blistering disease Pemphigus vulgaris (PV), intercellular adhesion is lost due to the disassembly of desmosomes which is triggered by the presence of autoantibodies against the desmosomal cadherin Desmoglein 3 (Dsg3). Plakophilin-1 (PKP-1) is a cytoplasmic regulator of desmosomes and is expressed in differentiated layers of stratified epithelia. Considerable overlap in the expression patterns of Dsg3 and PKP-1 exists, but the functional relationship between these two proteins is not well defined. To investigate the role of PKP-1 in PV and Dsg3 regulation, primary human keratinocytes were infected with adenoviruses carrying full length PKP-1 and then incubated with PV IgG or normal human IgG. Immunofluorescence microscopy of fixed cells and time lapse imaging of living cells revealed that PKP-1 blocked Dsg3 and desmoplakin (DP) disruption and prevented keratin retraction in response to PV IgG. Furthermore, in functional assays, PKP-1 prevented the loss of cell adhesion strength caused by PV IgG. To determine how PKP-1 regulates Dsg3 adhesion strength, PKP-1 was co-expressed with chimeric proteins that harbor either full length or truncated versions of the Dsg3 cytoplasmic tail fused to the extracellular domain of the interleukin-2 receptor (IL-2R). Immunofluorescence and co-immunoprecipitation experiments indicate that the Dsg3 cytoplasmic tail confers a specific interaction with PKP-1 and that this interaction requires the plakoglobin (PG) binding site within the Dsg3 tail. PKP-1 also caused co-clustering of both endogenous Dsg3 and the IL-2R-Dsg3 full length chimera, and these clusters recruited other desmosomal components. However, PKP-1 did not associate with or co-cluster the IL-2R-Dsg3 chimera lacking the PG binding domain. These data indicate that PKP-1 interacts with Dsg3 and reinforces keratinocyte adhesion through protein interactions that require the plakoglobin binding domain of the Dsg3 tail, and demonstrate a key role for desmosomal plaque proteins in regulating desmosome adhesive strength and sensitivity to PV IgG.

2033/B412
Regulation of Intercellular Adhesion: Regulatory Role of Catenins in Single E-Cadherin/E-Cadherin Bond Interactions.
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Cell-cell adhesion is a tightly regulated process essential for healthy tissue architecture whereas aberrant cell-cell adhesion has been implicated in several pathologic conditions. We postulate that E-cadherin induced cell-cell adhesion exhibited by epithelial tissues is modulated, on a single-molecule basis, by altered interactions between E-cadherin cytoplasmic tail and catenins. Using single-molecule force spectroscopy on live E-cadherin expressing cells, we show that when α-catenin is bound to the cytoplasmic tail of E-cadherins, the mean E-cad/E-cad bond strength is
significantly higher than inn cells lacking α-catenin. Moreover, the presence of α-catenin in adhesion complex ensures time-dependent maturing of a single E-cad/E-cad bond. The distribution of single-molecule bond-ruptures shows that E-cad/E-cad bonds are significantly more stable when α-catenin is bound to the cytoplasmic tail, as compared to when α-catenin is not bound. Likewise, recent data shows that β-catenin acts as a regulator of E-cadherin induced adherens junctions. These single-molecule force measurements correlate broadly with the strength of global-adhesion as exhibited by test-cells, suggesting that both avidity and affinity between E-cadherins expressed on adjacent cells, change in a related manner. Together, these results suggest that the extracellular cell-cell adhesion is regulated by intracellular protein-protein interactions on a single-molecule basis.

2034/B413
The Desmosomal Cadherins, Desmoglein 2 and Desmocollin 2, Rely on Distinct Mechanisms for Delivery to the Plasma Membrane.
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The desmosomal cadherins, desmogleins (Dsg) and desmocollins (Dsc) make up the adhesive core of intercellular junctions known as desmosomes, which play critical roles in tissue integrity. However, mechanisms that coordinate their trafficking into intercellular junctions to regulate their proper ratio and distribution on the cell surface are unknown. Using live cell imaging we show that both desmosomal cadherins undergo rapid, long range MT-dependent trafficking in epithelial cells. However, Dsg2 and Dsc2 exhibit distinct vesicular distribution patterns and dynamics, with Dsg2 vesicles having slower average maximum velocity and shorter trajectories. As antibodies cross-reacting with kinesin-1 co-localized with Dsg2-containing vesicles, an shRNA construct was introduced into keratinocytes to knock-down the kinesin-1 heavy chain (KHC), followed by examination of steady state and cell contact-initiated dynamics of this cadherin. Fluorescence intensity measurements revealed that Dsg2 at cell-cell interfaces of KHC-deficient cells was reduced to less than 50% under both conditions. Further, Dsg2 vesicle movement was largely blocked in cells expressing the shRNA or a dominant negative mutant of kinesin-1 without affecting the movement of Dsc2 or accumulation of Dsc2 or E-cadherin into intercellular junctions. Interestingly, knock down of the desmosomal cadherin-associated protein plakophilin 2 (PKP2) also inhibited Dsg2 accumulation in desmosomes, and PKP2 was observed to co-immunoprecipitate with kinesin-1. Thus, the temporal and spatial coordination of Dsg2 and Dsc2 assembly into intercellular junctions is controlled by distinct mechanisms involving kinesin motors, possibly mediated via associated armadillo proteins. These data have important implications for the regulation of intercellular adhesion in tissue homeostasis and remodeling, as well as during pathogenesis of desmosome-related diseases of the skin and heart.

2035/B414
The Desmosomal Cadherin Desmoglein 1 Interacts with Erbin: A Potential Mechanism by Which Desmoglein 1 Modulates Egfr/Erbb2 Signaling to Drive Epidermal Differentiation.
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Desmogleins and desmocollins are cadherins comprising the adhesive interface of desmosomes and are essential to the integrity of tissues enduring mechanical stress. In epidermis, desmoglein 1 (Dsg1) is required to maintain adhesion among differentiated keratinocytes in the upper layers; however, Dsg1 is expressed early in epidermal differentiation as growth factor signaling is dampened and cells begin to stratify. We recently demonstrated that RNAi-mediated silencing of Dsg1 in an organotypic model of epidermis resulted in profound defects in differentiation. Dsg1 reconstitution restored differentiation despite removal of ectodomain residues required for adhesion or uncoupling of the cadherin from its catenin partner, plakoglobin. These findings
indicate that Dsg1 drives differentiation independent of its classical functions in trans-adhesion and catenin binding. Thus, we hypothesized that Dsg1 supports pro-differentiation signaling via novel interactions with its cytoplasmic region, which extends well beyond the intracellular catenin binding segment (ICS), where classical cadherins terminate. To identify novel cytoplasmic partners of Dsg1, we carried out a yeast two-hybrid CytoTrap screen, which revealed an interaction with erbin, an erbB2 interacting protein able to modulate Erk signaling. Full-length Dsg1 and erbin co-distributed in keratinocytes and were found in a biochemical complex; however, truncation of Dsg1 after the ICS prohibited this interaction. Interestingly, Dsg1 knock-down increased activation of EGFR/erbB2 along with the downstream Raf-Mek-Erk cascade and chemical inhibition of this aberrant signaling rescued differentiation. Similar to Dsg1 deficiency, erbin silencing inhibited differentiation in an Erk-dependent manner. Moreover, knock-down of erbin reduced Dsg1’s ability to induce certain markers of differentiation, supporting a functional connection between erbin and Dsg1. In summary, while Dsg1 plays an essential role in tissue integrity, our data demonstrate a novel cytoplasmic interaction with erbin, a key regulator of growth factor signaling. Thus, we propose a model in which Dsg1 and erbin facilitate epidermal differentiation via cooperative modulation of EGFR/erbB2 signaling.

2036/B415
Cadherin-23, an Atypical Cadherin, Is Localized to Sites of Heterotypic Cell-Cell Adhesion in a Model of the Tumor Microenvironment.
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In the early stages of breast cancer metastasis, epithelial tumor cells penetrate the basement membrane and invade the surrounding stroma, where they encounter stromal fibroblasts. Paracrine signaling between fibroblasts and epithelial tumor cells contributes to the metastatic cascade, but little is known about the contribution of adhesive contacts between these two cell types to metastasis. Here we show that MCF-7 breast cancer epithelial cells and normal breast fibroblasts make heterotypic cell-cell adhesions when grown in co-culture. We used RT-PCR to probe for the expression of cadherin family members by each cell type grown alone and in a co-culture model system and found that both cell types express a variety of cadherins, including E-, P- (in MCF-7s), N-, OB- (in HFs) and Cadherin-23. Immunoblotting of lysates from mono- and co-cultures shows that of the expressed cadherins, only Cadherin-23 is upregulated by co-culture, and immunocytochemistry suggests that Cadherin-23 is recruited to heterotypic adhesions. Cadherin-23 is an atypical cadherin with 27 extracellular cadherin repeats and a cytoplasmic domain with little homology to that of classical cadherins. Cadherin-23 shows sequence homology to members of the FAT and Dachsous families and has been shown to play a role in stereocilia structure and function in the cochlea and may play a role in the retina as well, but no other functions have been identified to date. These data suggest that Cadherin-23 may play a role in the earliest stages of metastasis by mediating heterotypic cell-cell adhesion in the tumor microenvironment.

2037/B416
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p120 catenin (p120) binds and stabilizes classical cadherins, making it a critical regulator of cell-cell adhesion. Unlike β-catenin, which is structurally related, few p120 binding partners have been identified and characterized. Here we report an efficient technique for identifying novel p120 binding partners that provides evidence of an interaction between p120 and the RhoA substrate p160 Rho Kinase (ROCK1). We have optimized several methods to generate a single-step immunoprecipitation approach for capturing weak and/or transient protein-protein interactions and identifying them by mass spectrometry (designated RCMSA for Reversible Crosslink Mass Spectrometry Analysis). Briefly, proteins are covalently crosslinked in situ using thiol-cleavable
DSP (Dithiobis[succinimidyl propionate]) or DTME (Dithio-bismaleimidoethane) chemistries and then recovered by immunoprecipitating p120. Binding partners are then selectively eluted by cleaving the cross-links with DTT (dithiothreitol), and identified by single-dimension liquid-chromatography tandem mass spectrometry. Crosslinking dramatically improved the efficiency of p120 co-immunoprecipitation with other members of the cadherin complex and revealed several new putative binding partners. Interestingly, one of these was the RhoA substrate p160 Rho Kinase (ROCK1). Using crosslinking, we showed that a fraction of ROCK1 binds to E-cadherin-bound p120 at cell-cell junctions. Similarly, ROCK1 and p120 clearly colocalized at cell-cell junctions, although the bulk ROCK1 was found in the cytoplasm, as described by others. Upon ROCK1 depletion by shRNA, p120 levels at adherens junctions were dramatically reduced, along with E-cadherin and other members of the cadherin complex. These data reveal that ROCK1 interacts physically with p120, and implies a role for this interaction in regulating cadherin stability.

2038/B417
Casapase-3 Cleavage Is Potentially Associated with Delta-Catenin’s Nuclear Functions.
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Delta-catenin is an Armadillo protein of the p120-catenin sub-class capable of modulating important intracellular processes such as cadherin stability, small GTPase activity and nuclear transcription. From screening a yeast two-hybrid library of human embryonic stem cell cDNA, we identified delta-catenin as a potential interacting partner of bait caspase-3, enzymatic activity of which plays essential roles in apoptosis, inflammation and stem cell differentiation. Delta-catenin’s association with caspase-3 was verified through In Vitro binding assays, while delta-catenin protein was cleaved by enzymatic active caspase-3 or in Xenopus apoptotic extracts. The cleavage site, a highly conserved caspase consensus motif (DELD) within Armadillo repeat 6 of delta-catenin, was identified through peptide sequencing. Cleavage thus generates an N- and C-terminal fragment each containing about half of the central Armadillo domain. Interestingly, the C-terminus (delta-C hereafter) possesses a conserved putative nuclear localization signal (GKKKKKKKSQ) that may facilitate delta-catenin’s nuclear targeting in defined contexts. To probe delta-catenin’s possible nuclear roles, we performed yeast two-hybrid screening of a mouse brain cDNA library. In addition to delta-catenin’s published interactions such as with cadherins and Erbin, we identified members of KRAB (krüppel-associated box) zinc-finger family. Such proteins feature a C-terminal zinc-finger region that binds DNA, and an N-terminal KRAB motif associating with transcriptional cofactors. Preliminary deletion mapping has indicated that delta-C is both sufficient and necessary for its interaction with zinc-finger region of the KRAB member tested. In the near future we will further characterize delta-catenin’s relationship with caspase-3, including its possible relevance to delta-catenin’s association and functions in the context of KRAB zinc-finger transcription factors.

2039/B418
Protein Tyrosine Phosphatase Ptp1b Is Required for P120 Catenin Association with N-Cadherin Precursor and Progress through the Secretory Pathway.
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Previous work demonstrates that PTP1B regulates positively N-cadherin-mediated adhesion, a function that implies direct binding of PTP1B to N-cadherin at the cell surface, and dephosphorylation of beta catenin bound to the complex. This event contributes to stabilize N-cadherin-cytoskeletal connections essential for adhesion. Here we report a novel role of PTP1B in N-cadherin precursor trafficking, which does not require previous PTP1B binding, and it is independent of its role in beta catenin dephosphorylation. Indeed, co-localization and co-immunoprecipitation analysis did not show evidence of PTP1B/N-cadherin precursor complexes.
N-cadherin precursor mutants which cannot bind PTP1B traffic normally from ER to Golgi. In contrast, PTP1B knockout (KO) cells, or wild type (WT) cells functionally impaired by dominant-negative constructs, show arrest of N-cadherin precursors in a pre-Golgi stage. This defect cannot be attributed to beta catenin dissociation from N-cadherin precursor complexes since similar levels of beta catenin are found in complexes isolated from WT and KO cells. Interestingly, KO cells showed reduced levels of p120 associated to N-cadherin precursor. Moreover, consistent with a direct role of PTP1B in p120 dephosphorylation, phosphotyrosine content of total p120 was significantly enhanced in KO cells. Expression of N-cadherin precursor mutant that cannot bind p120 in WT cells is significantly arrested in a pre-Golgi stage. Also, a significant accumulation of N-cadherin-GFP occurs in CHO-K1 cells co-transfected with small constructs that sequester endogenous p120. Our results suggest that ER-bound PTP1B promotes trafficking of the N-cadherin precursor through a mechanism that requires its association with p120. Work supported by ANPCyT and CONICET.

**2040/B419**

**The C-Terminus of Desmoglein-2 Regulates Intestinal Epithelial Cell Proliferation By Activating β-Catenin/TCF Transcription.**

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The desmosome (DM) is an intercellular junction that is essential for maintaining tissue integrity. In simple epithelia DMs consist of the trans-membrane DM cadherins, desmoglein-2 (Dsg2) and desmocollin-2 (Dsc2), which are linked to cytokeratins via plaque proteins. Desmosomal proteins have been shown to regulate cell growth and morphogenesis, but the underlying mechanisms are not known. To better understand the biological function of DM cadherins in simple intestinal epithelia, siRNA was used to down-regulate Dsc2 expression in a model intestinal epithelial cell line SK-CO15. Using this approach, we observed that loss of Dsc2 resulted in enhanced cell proliferation and decreased membrane localization of beta-catenin, a known regulator of intestinal cell proliferation. Beta-catenin is a well-described component of adherens junctions (AJ). Using immunoblot analysis, we observed that down-regulation of Dsc2 did not influence total protein levels or distribution of other key AJ proteins, E-cadherin and p120. In addition, total levels of the DM proteins desmoplakin and plakoglobin were unchanged. Notably, we observed that Dsc2 downregulation resulted in increased cleavage of the Dsg2 cytoplasmic tail. The increase in Dsg2 cleavage was prevented by treatment with an inhibitor of cysteine proteases, suggesting that loss of Dsc2 enhances the susceptibility of Dsg2 to cleavage by cysteine proteases. Furthermore, our data demonstrates that the Dsg2 cleavage fragment positively regulates beta-catenin signaling and intestinal epithelial cell proliferation. Thus, we speculate that Dsc2 functions to suppress intestinal cell proliferation by stabilizing and preventing the cleavage of Dsg2, thereby inhibiting beta-catenin signaling.

**2041/B420**

**Zebrafish Maternal Zygotic dachsous1 Mutants Have Defects in Early Embryogenesis.**

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Cadherins are calcium dependent adhesion molecules and can also be involved in cell signaling. Unlike classical cadherins, protocadherins do not have connections to the cytoskeleton through catenins. Dachsous (Dchs) is a protocadherin with 27 extracellular cadherin repeats, a transmembrane domain, and an intracellular domain. Studies in Drosophila have indicated the involvement of Dachsous in planar cell polarity (PCP) of epithelia as well as organ size control. Studies of the mammalian Dchs homologs have shown that along with the four fat proteins (another protocadherin), Dchs regulate apical membrane organization through cell-cell adhesion. Zebrafish, with large clutches, transparent embryos that develop externally, and effective genetic tools is an excellent model to study conserved pathways in vertebrate development. In zebrafish, as in humans, there are two dachsous genes. Through targeting induced local lesions in genome (TILLING), a high throughput method to detect mutations in specific genes as described by
Wienholds et al. 2002, two nonsense mutations in dchs1 have been identified. Zygotic mutants show no obvious phenotype. Because dchs1 is expressed maternally, we generated mutants lacking both its maternal and zygotic functions (MZdchs1). These MZdchs1 mutant embryos show defects in cell adhesion, cell division, and morphology at the cleavage stages. At later stages during gastrulation, these embryos have delayed epiboly; and even later during segmentation period, they show delayed organogenesis. However, as development progresses, these defects subside and by 24 hours post fertilization (hpf), the mutants closely resemble wild-type embryos. Although the survival rate of these mutants is lower than wild type, they do survive to adulthood and can reproduce. These preliminary data indicate an important role for dachsous1 in early embryonic development.

2042/B421
Expression of p120-Catenin Is Post-Transcriptionally Regulated By Cdk5.
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Purpose: Inhibition of Cdk5 reduces cell-cell adhesion and promotes migration of wounded corneal epithelial cell sheets. This study investigates the role of Cdk5 in maintaining cell-cell adhesion by examining expression and localization of junctional components in a human corneal epithelial cell line. Methods: Cdk5 activity was blocked by lentiviral vector mediated stable expression of Cdk5 shRNA or by 15μM olomoucine. Expression and localization of junctional components were examined by immunoblotting, co-immunoprecipitation, immunofluorescence, and total internal reflection fluorescence (TIRF) microscopy. RNA expression was determined by RT-PCR. Surface-exposed E-cadherin was labeled by biotinylation and isolated by affinity chromatography on streptavidin-coated beads. Results: Inhibiting Cdk5 activity or suppressing Cdk5 expression increased expression of p120-catenin two fold with no corresponding increase in p120 mRNA. E-cadherin expression decreased slightly, while expression of beta-catenin and alpha-catenin were not significantly changed. Accumulation of p120 produced by Cdk5 inhibition was accompanied by numerous signs of junctional instability. The half-life of surface-biotinylated E-cadherin decreased markedly; the proportion of total E-cadherin localized at cell-cell boundaries decreased; and the co-immunoprecipitation of IQGAP with E-cadherin Beta-catenin increased; finally, TIRF microscopy showed increased internalization of vesicles containing E-cadherin. No corresponding internalization of p120 was observed, indicating that internalized E-cadherin is not associated with p120. Conclusions: Expression of p120-catenin is post-transcriptionally regulated by Cdk5. Regulation may involve direct phosphorylation of favorable sites by Cdk5, followed by targeted degradation of p120. Accumulation of p120 is accompanied by decreased junctional stability, evidenced by degradation of surface-labeled E-cadherin, a shift in E-cadherin localization from cell-cell boundaries to the cytoplasm, and increased association of E-cadherin Beta-catenin with IQGAP. Excess p120 may participate in destabilizing cell-cell junctions through its known role in regulating Rho-GTPases.

2043/B422
Differential Regulation of Cell Motility by Desmosomal versus Classical Cadherin.
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During normal development and cancer, epithelia undergo various shape transformations that range from cohesive sheet migration, to cell position changes within cohesive tissues, to individual cell migration after dissociation. Such cell movements require coordinated cross-talk between cadherins, involved in cell-cell interactions, and integrin adhesion receptors to the extracellular matrix (ECM), involved in cell migration. Epithelial cells can form two types of cell-cell adhesions by members of the cadherin superfamily: desmoglein (dsg) and desmocolin (dsc) form desmosomes and E-cadherin (Ecad) forms adherens junctions. The respective contributions of these adhesion complexes to the adhesive properties of cells and their subsequent motile behavior in tissues is unknown. We used functionalized micro-patterned surfaces comprised of
alternating stripes of collagenIV and either dsg/dsc or Ecad to analyze the effects of cross-talk between these adhesion receptors on individual cell behaviors. We show that desmosomal cadherin adhesion dramatically reduces cell migration rate, possibly through the formation of shear-resistant cell adhesion to the dsg/dsc surface, although membrane activity at collagenIV contacts remains active. In contrast, E-cadherin adhesion suppresses membrane activity on both E-cadherin and collagenIV surfaces, but does not affect the rate of cell migration. We suggest that the desmosomal cadherin adhesion is more rigid, resulting in reduced cell migration rate and shear-resistant epithelium. Conversely, E-cadherin junctions provide fluid contacts that can maintain tissue cohesion while allowing movement of cells past each other and en masse sheet migration. These differences between the cell-cell adhesion complexes may contribute to the range in morphogenetic cell movements.

2044/B423  
Protocadherin-19 Acts Synergistically with N-Cadherin during Early Zebrafish Brain Morphogenesis.  
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The vertebrate brain is a structure of remarkable complexity. However, the cellular and molecular mechanisms by which the mature brain is sculpted during development remain in completely understood. We have identified a member of the cadherin superfamily, protocadherin-19 (pcdh19), that plays an essential role in early zebrafish brain morphogenesis. In humans, mutations in pcdh19 are responsible for an X-linked, female limited form of epilepsy and mental retardation (Epilepsy and mental retardation limited to females, EFMR). Knockdown of pcdh19 in zebrafish using morpholinos results in defects in neurulation specific to anterior regions of the neural plate. Loss of Pcdh19 function impairs convergence movements, resulting in aberrant brain morphology. The observed phenotypes are similar to those exhibited in N-cadherin (Ncad) mutant and morphant embryos, suggesting a potential interaction between Pcdh19 and Ncad. We are using In Vivo 2-photon time-lapse imaging and quantitative analysis of cellular movements during neurulation to characterize the defects in Pcdh19 and Ncad morphant embryos. Using co-immunoprecipitation, we find that Pcdh19 and Ncad interact physically, when co-transfected into heterologous cells in vitro. Similarly, these two proteins co-localize, as assessed by immunofluorescence microscopy. We also have evidence that Pcdh19 and Ncad collaborate in vivo, during neurulation. Injection of sub-threshold doses of morpholinos directed against Ncad or Pcdh19 result in no observable phenotype. However, co-injection of these low doses results in a robust phenotype similar to those observed with full doses of either Ncad or Pcdh19 morpholino alone. These data suggest a genetic interaction, with Pcdh19 and Ncad acting in a linear or in parallel pathways during neurulation. We are using both In Vivo and In Vitro approaches to characterize the interaction of Pcdh19 with Ncad in more detail.

2045/B424  
Investigations of Single Protocadherin Isoforms in the Developing Zebrafish.  
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The clustered protocadherins comprise the largest subfamily of protocadherins (pcdhs). They have stimulated much interest as potential synaptic specificity molecules due to their neuronal expression and notable genomic organization into 5’ variable exons that are spliced onto 3’ constant exons. Each variable exon is expressed under the control of its own promoter, which is then spliced to the constant exons to generate a full-length protein. There are two classifications of clustered protocadherin isoforms: V-types and C-types. C-type protocadherins are distinct from V-types in that they are ubiquitously- and biallelically-expressed, and are more highly conserved between species. Our preliminary studies on the pcdh1alpha cluster, as a whole, revealed that loss of pcdh1alpha activity results in a dramatic increase in programmed cell death throughout the nervous system. We also characterized the dynamic distribution of the Pcdh1alpha-GFP
fusion protein, which is concentrated in the growth cone in discrete, mobile puncta within axons, and in puncta at the tips of filopodia. We have begun investigating the functional role of single isoforms of alpha-protocadherins in the developing zebrafish. Zebrafish have two Pcdh-alpha clusters, termed Pcdh1alpha and Pcdh2alpha, due to genomic duplication. Each cluster has one C-type isoform: Pcdh1alpha-10 and Pcdh2alpha-35. In the current set of studies, we have concentrated on the single C-type isoform Pcdh1alpha-10. We have demonstrated effective knockdown of the single Pcdh1alpha-10 isoform using morpholinos along with Western blot analysis and RT-PCR. We are using TUNEL assays and whole-mount immunocytochemistry, as well as two-photon time-lapse analysis, to characterize the effects of Pcdh1alpha10 knockdown on nervous system development. In addition, we have used BAC recombineering to tag the full-length Pcdh1alpha10 as well as a series of deletion mutants, expressing the gene fusions under the control of the native promoter. This approach will allow us to assess the distribution and dynamics of Pcdh1alpha-10 and mutants within neurons in vivo, as well as their effects on growth cone and filopodia dynamics.

2046/B425
A Cyclic Amp-Epac-Rap1 Pathway Induces Formation of Circumferential Actin Bundles, Which Stabilize Junctional Ve-Cadherin through Alpha-Beta-Catenins.
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Vascular endothelial-cadherin (VE-cad) is a cell-cell adhesion molecule involved in the formation of endothelial adherens junctions. Previously, we have reported that cyclic AMP (cAMP) enhances VE-cad-dependent cell adhesion through an Epac-Rap1 pathway. Here, we further scrutinized the molecular mechanism by which cAMP promotes VE-cadherin-based cell-cell adhesions. Forskolin (FSK; an adenylyl cyclase activator) induced circumferential actin bundling along endothelial cell-cell borders and accumulation of VE-cad and VE-cad carboxy-terminally fused with GFP (VEC-GFP) on the bundled actin filaments. Fluorescence recovery after photobleaching analysis using VEC-GFP revealed that FSK stabilized VEC-GFP at the cell-cell contacts. Stability of junctional VEC-GFP was also enhanced by a cAMP analog for Epac, but not by that for protein kinase A. FSK-induced stabilization of VEC-GFP at cell-cell junctions was prevented by latrunculin A, an inhibitor for actin polymerization. Furthermore, a VEC-GFP mutant lacking beta-catenin (ctn) binding site and that lacking cytoplasmic domain of VE-cad exhibited less accumulation and stabilization at the cell-cell contacts in FSK-stimulated cells. However, FSK induced accumulation and stabilization of a VEC-GFP mutant, in which cytoplasmic domain of VE-cad was replaced with alpha-ctn, at cell-cell contacts. Collectively, these results suggest that a cAMP-Epac-Rap1 pathway initially induces formation of circumferential actin bundles along cell-cell junctions, which anchor VE-cad through alpha- Beta-ctn, thereby stabilizing VE-cad-based cell-cell adhesions.

Cell-Cell Adherens Junctions (2047 – 2062)

2047/B426
A Putative Zyxin-Nectin Interaction at Cell-Cell Contacts.
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Zyxin localizes to sites of cell-cell adhesion, where it participates in linking the actin cytoskeleton to the adherens junction membrane. Zyxin binds to actin filaments using amino terminal proline-rich motifs that bind actin regulatory proteins of the VASP family. Zyxin’s association with the adherens junction membrane is less well defined. Several zyxin fragments have been shown to localize independently to cell-cell contacts, including carboxyl terminal LIM domains, the extreme amino terminal alpha-actinin binding site, and the central region between the proline-rich motifs and the LIM domains. Since zyxin LIM domains can interact directly with VASP and since zyxin is required for VASP localization to cell-cell adhesions, the LIM domains likely do not drive zyxin’s cell-cell junction localization. It has been previously proposed that zyxin localizes to cell-cell
contacts via an interaction with alpha-actinin. However, deletion of the alpha-actinin binding site from a constitutively active zyxin mutant did not result in its loss of cell-cell junction targeting nor its loss of function in driving accelerated cell-cell junction formation. Here we analyze the central region of zyxin to identify the specific region that mediates zyxin localization to cell-cell contacts and attempt to identify binding partners for this region. Progressive deletions from the carboxyl terminus of a zyxin fragment reveal that cell-cell junction targeting in the central region of zyxin is located in amino acids 280-330. Few binding partners for the central region of zyxin have been identified, and none for the specific region characterized here. We generated a yeast two hybrid bait containing the zyxin central region and screened for binding partners. Among candidate binding partners identified was nectin-4, a cell-cell adhesion molecule. Nectins mediate homotypic and heterotypic interactions in their extracellular domains and become linked to the actin cytoskeleton via their intracellular domain binding partners. Our results indicate that zyxin could provide a new mechanism for linking nectin-based adhesions to actin filaments, as well as allowing nectin-based adhesion to drive actin remodeling through VASP family members.

2048/B427

The Polarity Protein Bazooka Is Differentially Phosphorylated during Epithelial Morphogenesis.

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The polarity protein Bazooka (Baz)/PAR3 is a key regulator in establishing and maintaining epithelial polarity, functioning as a primary landmark for adherens junction (AJ) positioning in Drosophila. As the first epithelium forms Baz localizes to the apical domain where it organizes preformed clusters of DE-Cadherin and Armadillo into precursory AJs. Although Baz plays an important role in AJ assembly, the molecular mechanisms regulating Baz function and cellular localization remain unclear. Many cellular processes are controlled by phosphorylation of critical functional components. Studies in vertebrates suggest that maintenance of AJ integrity is regulated, in part by, reversible phosphorylation events. Baz has previously been shown to be serine phosphorylated and mammalian PAR3 is phosphorylated on both serine and tyrosine residues, but how these modifications affect the interaction of Baz with AJs is not known. Using immunoprecipitation and western blot analysis, we show that Baz is tyrosine phosphorylated in Drosophila embryos. Phosphorylated Baz is detected using phosphotyrosine (pY) antisera and this phosphorylation is lost upon treatment with calf intestine phosphatase. To roughly map the sites of Tyr phosphorylation we examined deletion mutants of Baz. Deletion of both the PDZ domains and C-terminus of Baz abolishes Tyr phosphorylation however mutants deleting the N-terminus or the PDZ domains are still Tyr phosphorylated suggesting phosphorylation is within this C-terminal region. Developmental time course analyses suggest Baz phosphorylation is differentially regulated at different stages of embryonic development with a peak in Tyr phosphorylation during gastrulation. Interestingly, Baz localizes to sites rich in pY within the apical domain of epithelial cells although pY also localizes to other regions. Overexpression of Baz appears to enhance its localization with pY. These data suggest that Tyr phosphorylation of Baz is specifically regulated over development and we speculate that Baz tyrosine phosphorylation depends on its localization in the cell. To understand the relevance of Baz phosphorylation, we are working to identify which tyrosine residues are phosphorylated and the kinases involved.

2049/B428

Positive Regulation of Endothelial Barrier Function by R-Ras and Filamin A.

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The vascular endothelium acts as a restrictive barrier that regulates many biological processes such as protein and fluid transport and inflammation. Disruption of this barrier leads to leakiness resulting in edema and organ dysfunction. The cellular mechanisms that regulate endothelial barrier function are not yet completely known. The membrane cytoskeletal protein Filamin a (FLNa) is required for cell-cell contact in vascular development and in cardiac morphogenesis.
Many cardiovascular defects are associated with the loss of human FLNa function such as periventricular heterotopia, cardiac valvular anomalies, and vascular disorders. FLNa-null mice die of vascular defects including dilated vasculature, which suggested to us alterations in vascular permeability. R-Ras, a small intracellular GTP-binding protein, is primarily expressed in endothelial cells In Vivo and is a regulator of vascular differentiation and remodeling of blood vessels. The objective was to investigate whether FLNa and R-Ras are positive regulators of endothelial barrier function. Endogenous R-Ras interacts in a complex with endogenous FLNa, pullows down with FLNa fragment repeats 1-10 and colocalizes at the plasma membrane in Human Coronary Arterial Endothelial Cells (HCAECs). Knockdown of R-Ras/FLNa using siRNA promotes endothelial barrier dysfunction via increased vascular permeability as determined by Transendothelial Electrical Resistance (TER) and FITC-dextran transwell assay. Knockdown of FLNa/R-Ras increased phosphorylation of Ve-cadherin (Y731) and immunostaining demonstrated a zipper-like disorganization of Ve-cadherin similar to TNFalpha-mediated inflammation. Because Ve-cadherin regulates c-src activation in response to growth factor-mediated inflammation we next examined c-src levels in HCAECs where FLNa/R-Ras had been knocked down. Indeed, we found that c-src expression increased in these cells in a similar manner as TNFalpha-inflamed cells. Our data suggest that maintaining endothelial barrier function may be, in part, dependent upon the interaction between R-Ras and FLNa and that loss of this interaction promotes changes in Ve-cadherin phosphorylation and downstream signaling that mediate vascular permeability.

2050/B429
Altered Expression of N-Cadherin and Beta-Catenin Precedes Gap Junction Remodeling during Development of Heart Failure.
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The intercalated disc contains different junctional complexes (adhesion junctions and gap junctions) that enable the myocardium to function as a syncytium. The adhesion junctions are organized to mediate normal mechanical coupling between cardiomyocytes and play a key role in the formation and stability of gap junctions. The remodeling of ventricular gap junction connexin43 (Cx43) is a prominent feature of arrhythmogenesis in diseased myocardium. To investigate alterations in adhesion junction proteins during the development of heart failure, we used UM-X7.1 cardiomyopathic hamster hearts. at age 10~15 wk (hypertrophic stage), 10% of cardiomyopathic hamsters died suddenly without heart failure, and VT/VF was inducible in 30% hamsters. Western blotting revealed that total N-cadherin expression was significantly increased at 15 wk and 20 wk (compensated heart failure stage), and total β-catenin expression was increased at 15 wk compared with age matched control. Immunohistochemical analysis revealed that both N-cadherin and β-catenin expression at intercalated disc were increased at 15 wk and 20 wk compared with age-matched control. The nuclear protein expression level of β-catenin, which is also known to translocate to the nucleus and increase TCF/LEF transcriptional activity, was remarkably decreased (~50% decrease) compared with age-matched control at age 10 wk. at age 20 wk, LV Cx43 expression was significantly decreased (~50% decrease) with a remarkable increase of Ser255-phosphorylated Cx43 expression. The lethal ventricular arrhythmias were inducible in all cardiomyopathic hamsters. These results suggest that a decrease of β-catenin expression level in nucleus precedes gap junction remodeling, resulting in decrease of Cx43 expression in LV of cardiomyopathic hamsters. In conclusions, changes of adhesion junctions might play an important role in the gap junction remodeling and contribute to an arrhythmogenesis in failing heart.

2051/B430
In Vivo Functional Analysis of Drosophila Alpha-Catenin.
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E-cadherin is a core component of adherens junctions (AJs) that together with p120-catenin, β-catenin and α-catenin forms the cadherin-catenin complex (CCC). Vertebrate analyses suggest that α-catenin allows the CCC to associate with the actin cytoskeleton to facilitate strong adhesion. To better understand how α-catenin supports cadherin activity during development, we use Drosophila as a model to carry out a detailed functional analysis of α-catenin. Null mutations of α-catenin were generated and consistent with the key function of α-catenin in cell adhesion, mutational analyses reveal that α-catenin deficient epithelial cells are unable to adhere to each other and loss of α-catenin in turn leads to embryonic lethality. α-catenin is related to the cytoskeletal protein Vinculin, and shows ~21-33% sequence identity at three regions previously annotated as Vinculin Homology (VH) regions - VH1, VH2 and VH3. Based on its sequence similarity with Vinculin and the comparison of α-catenin proteins from different species, we have generated deletion constructs that remove several conserved regions within α-catenin. Our rescue analyses show that both VH1 and VH3 (regions required to mediate interactions with β-catenin and actin, respectively) are essential for α-catenin function. The central VH2 region is required to stabilize α-catenin at the AJs but is not essential for α-catenin function. The linker region between VH1 and VH2 and the C-terminal region make minor contributions to α-catenin functions. Using a positive marking system to rescue α-catenin mutant epithelial cell in the follicular epithelium during oogenesis we found that deletion of VH1, but not VH2 or VH3, disrupts the localization of β-catenin at AJs. Deletion of VH3 supports the initial formation of cell-cell contacts but compromises follicular epithelium integrity. Our observations also suggest that the VH2 region modulates the strength of the binding between β-catenin and α-catenin. Analysis of additional constructs provides evidence suggesting that the rescue ability of α-catenin transgenes correlates with the strength of the interaction between α- and β-catenin.

2052/B431
Phosphorylation Regulates the Zyxin Head-Tail Interaction.
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Zyxin localizes to sites of actin-membrane interface, particularly cell adhesions, where it directs actin remodeling by binding to actin regulatory proteins of the VASP family. This interaction occurs at a series of proline-rich motifs near the amino terminus of zyxin. VASP binding to the proline-rich motifs is limited in full-length zyxin, but not in a zyxin fragment lacking carboxyl terminal LIM domains. We previously showed that purified full-length zyxin is co-immunoprecipitated with a purified zyxin LIM domain region fusion protein, except when antibodies targeted against the proline-rich motifs are used. These data show that the zyxin LIM domain region mediates a head-tail interaction that occurs at or near the proline-rich motifs, consistent with this interaction limiting zyxin-VASP binding. Further demonstrating a head-tail interaction, two binding partners of zyxin’s LIM domains, acinus-S and H-Warts/LATS1, have been demonstrated to have limited binding to full-length zyxin. Interestingly, zyxin phosphorylation confers the ability of LIM domain binding partners to associate with full-length zyxin, suggesting that zyxin phosphorylation might alter the head-tail interaction. The binding of acinus-S, in particular, appears to be regulated by zyxin phosphorylation at serine 152. Here we directly examine the effect of zyxin serine 152 phosphorylation on the head-tail interaction. Consistent with phosphorylation regulating the zyxin head-tail interaction, point mutations at serine 152 alter the ability of a zyxin fragment lacking LIM domains to bind a zyxin LIM domain region fusion protein. More specifically, a zyxin mutant bearing an alanine substitution at serine 152 retains the ability to bind the LIM domain fusion protein, while a zyxin mutant bearing a phosphomimetic glutamate substitution at serine 152 no longer binds the LIM domain fusion protein. In related work we showed that deletion of zyxin LIM domains generates a constitutively active mutation that affects cell-cell adhesion, suggesting that cells control zyxin function at adhesion sites thorough the LIM domains. Data presented here provides a molecular mechanism for such control.

2053/B432
Adducins Regulate Remodeling of Adherens Junctions in Human Epithelial Monolayers.
Epithelial adherens junctions (AJs) and tight junctions (TJs) are dynamic structures readily undergoing disintegration and reassembly during normal epithelial morphogenesis and in pathological conditions. Such junctional remodeling depends on orchestrated dynamics of the plasma membrane and the underlying actin cytoskeleton, which implicates the membrane/cytoskeleton interface in AJ/TJ regulation. The plasma membrane is linked to the actin cytoskeleton by a spectrin-adducin polymeric lattice. The aim of this study was to investigate the roles of adducin in the remodeling of epithelial AJs and TJs. To do that, we used SK-CO15 human colonic and HPAF-II human pancreatic epithelial cell monolayers, which possess well-formed apical junctions and express α- and γ isoforms of adducin. Both adducin isoforms colocalized intracellularly with E-cadherin and β-catenin in cell contact-naïve calcium-depleted SK-CO15 cells and rapidly accumulated in E-cadherin-based AJs during junctional reassembly. siRNA-mediated downregulation of α- and γ-adducin expression attenuated reformation of AJs without affecting TJ reassembly. Depletion of adducins in SK-CO15 cells decreased protein level of βII spectrin and downregulation of βII spectrin expression mimicked effects of adducins knockdown on AJ reassembly. Furthermore, adducin-depleted epithelial cells demonstrated defects in assembly of the perijunctional F-actin belt. Exposure of HPAF-II cells to a protein kinase C-activating phorbol ester resulted in rapid AJ/TJ disassembly accompanied by phosphorylation of adducins and their disappearance from intercellular contacts. Downregulation of α- and γ adducins accelerated phorbol ester-induced junctional disassembly. These finding suggest novel roles for adducins in the establishment and stabilization of epithelial adherens junctions.

2054/B433  
Rho GTPase-Activating Protein Myosin IXA Regulates Cell-Cell Contact Formation in Bronchial Epithelial Cells.  
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Embryonic morphogenesis and cancer invasion depend on collective cell migration. Cell-cell adhesion and anterior-posterior cell polarization are fundamental steps required for collective cell migration. We used bronchial epithelial cells 16HBE, which show remarkable coordinated migratory behavior in vitro, and an siRNA-based screen approach to identify components of Rho GTPase signaling pathway involved in collective cell migration. Silencing of myosin IXA, an unconventional myosin with Rho GTPase-activating protein (GAP) activity, led to inhibition of wound closure, which was associated with cell-cell contact disruption and random migration. We examined cell-cell collisions by high resolution time-lapse microscopy and found that myosin IXA siRNA-treated cells were unable to form cell-cell adhesions. Swellings and ruffles observed in the free edge lamellipodia of myosin IXA-depleted cells prevented lamellipodia overlapping leading to cell-cell retraction upon collision. Rho kinase inhibitor treatment prevented formation of swelling and partially reversed the observed phenotype. Rat myosin IXA rescued cell-cell integrity in myosin IXA siRNA-treated cells. Cloned and ectopically expressed human EGFP-Myosin IXA localized to cell-cell contacts in an F-actin-dependent manner. These findings suggest that myosin IXA may function to downregulate Rho signaling to allow lamellipodia to overlap and stabilize actin bundles in response to cadherin ligation.

2055/B434  
Involvement of Talin in Cell-Cell Adhesion by a Novel C-Terminal Cleavage Product.  
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Talin is an essential protein involved in cell migration and adhesion that is known as the linker between the intracellular actin networks and the transmembrane protein integrin. Our recent study showed that talin is subjected to posttranslational arginylation on Ala 1903, suggesting the existence of a previously unknown proteolytic processing, yielding a ~70 kDa fragment from Ala 1903 to the C-terminus of talin, predicted to contain the vinculin, integrin and actin binding domains (VIA fragment). Here we demonstrate the In Vivo generation of the predicted talin via
fragment and find that the existence of this fragment is dependent on calpain activity, arginylation activity, and cell density in culture. Remarkably, further analysis shows that the generation of the via fragment is specifically associated with the formation of cell-cell adhesions, providing an unexpected functional link between talin and cell-cell contacts. Antibody staining and GFP fusion construct studies show that the via fragment, but not the full length talin, localizes to the cadherin-positive punctae at the cell-cell contact sites that precede the formation of the mature cell-cell adhesions. Arginylation-deficient cells, in which the generation of via fragment is inhibited, appear to have strong defects in cell-cell adhesion. Our data suggest a novel mechanism that links talin to cell-cell adhesion formation through generation of a C-terminal truncate that is regulated by arginylation. This mechanism likely coordinates the formation of actin networks in the nascent cadherin-positive cell-cell contacts.

2056/B435
Alpha-Catenin Regulation of Actin Dynamics and Cell-Cell Adhesion.
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The development of multicellular organisms requires the orchestrated movement and adhesion of cells that are driven by the regulated assembly and organization of actin networks. Cadherins are key regulators of cell-cell adhesion and are associated with the actin cytoskeleton primarily through α-catenin, which binds the cadherin/β-catenin complex and F-actin. In Vitro studies indicated that the oligomeric state of α-E-catenin, the epithelial form of α-catenin, modulates its binding affinities: the monomer forms a ternary complex with β-catenin and E-cadherin, whereas the homodimer preferentially binds to actin filaments and inhibits Arp2/3-mediated actin polymerization. To investigate how α-catenin regulates actin dynamics underlying cell-cell adhesion and cell motility, the cytosolic pool of α-E-catenin was sequestered to mitochondria or the plasma membrane without affecting α-E-catenin levels or the strength of cadherin-mediated cell-cell adhesion. Mitochondrial sequestration of α-E-catenin increased cell migration, lamellipodial dynamics, cortical actin filament polymerization and branched-actin organization. In contrast, recruitment of α-E-catenin to the plasma membrane decreased membrane dynamics, cortical actin polymerization and branched-actin structures. Thus, α-E-catenin functions in cell-cell contact dependent and independent modes to regulate actin dynamics during epithelial cell-cell adhesion and cell migration. In contrast to α-E-catenin, the highly related neuronal form α-N-catenin is predominantly monomeric at concentrations (≈10 μM) that promote α-E-catenin homodimerization. Functional complementation tests conducted in MDCK cells, in which endogenous α-E-catenin expression was silenced using RNAi knockdown, revealed that expression of α-N-catenin rescues cadherin-mediated cell-cell contact formation and cell-cell adhesion defects. Thus, homodimerization does not appear to be required for α-catenin function in cell-cell adhesion. Together, these experiments define new functions for α-catenin and offer further insight into the role of α-catenin in cadherin-mediated adhesion.

2057/B436
Scribble and Met Receptor Kinase Signaling Complex.
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The activity or levels of Met, a receptor tyrosine kinase binding hepatocyte growth factor, are frequently elevated in human cancers. Upon Met receptor stimulation, many epithelial cells in culture recapitulate the behavior of metastatic cells: cell-cell adhesion is lost, and cells scatter or migrate in 2D, invade though matrix and undergo an invasive morphogenic process in 3D, a process reminiscent of epithelial organogenesis or angiogenesis. These processes require dynamic remodeling of epithelial cell polarity. However, how Met signaling elicits these changes in cellular morphology required for the metastasis-like behavior of cells is still largely unknown. By
co-immunoprecipitation, we identified Scribble, a conserved scaffold protein required for cell-cell adhesion, cell migration and cell polarity as interactor in the Met receptor signaling complex. Scribble acts as a tumor suppressor in flies and mammalian systems and reduced levels or mislocalization of Scribble protein are observed in many human cancers. We show that Scribble localizes, like the Met receptor tyrosine kinase, to the basolateral membrane in polarized epithelial cells. Biochemical analysis indicates that the interaction between Scribble and Met is independent of maturation or activation of the receptor and does not require the Met scaffold protein Gab1. Given the versatile role of Scribble in the organization of cell polarity and its implication in tumor suppression, we hypothesize that Scribble acts in Met signaling, either upstream or downstream of the Met receptor, contributing to the changes in cellular organization observed during Met-induced signal transduction. We are currently testing these possibilities using immunofluorescence and different cell-based assays.

2058/B437
Dissecting the Relationship between p120-Catenin Binding to E-Cadherin Juxtamembrane Domain, and the Ubiquitination and Degradation of E-Cadherin.
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E-cadherin, a component of the Adherens Junction, initiates the formation of cell-cell contacts. E-cadherin stability is necessary to maintain adhesive contacts during tissue development, but is modified during tissue reorganization to allow increased cell dynamics. p120-catenin (p120) binds to the juxtamembrane domain (JMD) of E-cadherin, and this interaction has been proposed to stabilize E-cadherin since uncoupling of this interaction induces endocytosis of E-cadherin. Upon endocytosis, E-cadherin may be recycled back to the membrane or degraded. The mechanism by which E-cadherin is degraded is unknown. To examine how p120 regulates E-cadherin stability and the mechanism of E-cadherin degradation, the JMD of murine E-cadherin, tagged with monomeric RFP, was targeted to mitochondria of MDCK cells by an ActA peptide sequence (JMD-ActA). In these cells, JMD-ActA localizes with mitochondrial markers and levels and location of endogenous plasma membrane cadherin-catenin complexes were unaffected. However, p120 did not accumulate at the mitochondria, and decreased amounts of JMD-ActA at the mitochondria were observed in comparison to a control vector (ActA) indicating that JMD-ActA is degraded. To assay for degradation, MDCK cells expressing JMD-ActA or ActA were incubated with proteasome inhibitor MG-132. Western Blot analysis of cell lysates demonstrated an increased level of JMD-ActA upon MG-132 treatment, but not ActA. RFP immunoprecipitations demonstrate that p120/JMD interaction was increased in the presence of MG-132, while inhibiting deubiquitinating enzymes (with NEM) reduced the interaction. p120 did not localize at mitochondria after MG-132 incubation. Mutation of lysine to arginine residues within JMD stabilized JMD-ActA at mitochondria and lead to recruitment of p120. HA tagged ubiquitin and FLAG tagged Hakai (ubiquitin E3 ligase) were expressed independently in JMD-ActA cells to observe the relationship of p120 binding and addition of ubiquitin. The data indicate that the JMD of E-cadherin is subject to degradation via the proteasome when p120 is not bound in an ubiquitin-dependent manner.

2059/B438
Role of p120-Catenin in Neutrophil Attachment and Activation.
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Barrier defects and excessive immune response in the intestine can lead to Inflammatory Bowel Disease (IBD). p120 catenin binds to the juxtamembrane domain of cadherins and modulates the strength of cell-cell adhesion by controlling E-cadherin stability at the cell surface. Targeted p120 knockout (KO) in salivary gland epithelium induces significant adhesive defects, whereas p120 KO in the epidermis leads to hyperplasia and chronic cell autonomous inflammation. We showed previously that conditional p120 KO in the intestine causes adhesive defects and massive
inflammation. To distinguish the relative roles of the barrier defect and postulated cell autonomous inflammation, we examined the effects of p120 KD In Vitro using the colon cell line HCA7. WT and p120 KD HCA7 cells were grown as polarized monolayers on neutrophil permeable filters and neutrophils were added to the basolateral side with chemoattractant (fMLP) on the other. Interestingly, 120 knock down induced complete loss of transepithelial electrical resistance (TER) irrespective of neutrophil presence. Neutrophil transmigration was unaffected, but attachment to the p120 KD monolayer increased 30 fold. Thus, p120 loss severely compromises barrier function, but also appears to induce a cell autonomous response that enhances neutrophil interaction with the epithelium. Longer exposure of neutrophils to the p120-deficient but not WT-monolayers resulted in increased neutrophil COX-2 staining. Our data reveal a potentially important role for p120 in regulating neutrophil attachment and possibly activation.

2060/B439
Rack1 Promotes Cell-Cell Adhesion and Suppresses Invasion of Colon Carcinoma Cells.
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The progression of epithelial tumors frequently involves destabilization of E-Cadherin- associated cell-cell adhesion, a consequence of mislocalization or loss of expression of E-Cadherin and associated Catenins. The tyrosine kinase, Src, is involved in both transcriptional and post-translational regulation of E-Cadherin and Catenins. Elevated Src activity, a prominent feature of colorectal and other epithelial cancers, is associated with aberrant growth control, disruption of cell-cell junctions, tumor dissemination and chemo-resistance. The adapter protein Rack1 is a non-catalytic, negative regulator of Src activity. Inhibition of Src by Rack1 at cell-cycle checkpoints decreases growth of colon cancer cells. Furthermore, Rack1 is involved in regulation of apoptosis, translation and migration by both Src dependent and independent mechanisms. The objective of this study is to assess Rack1 function in cell-cell adhesion of colon epithelial cells. Our results indicate that Rack1 facilitates E-Cadherin-dependent cell-cell adhesion and decreases matrigel invasion of colon carcinoma cells, partly via its ability to inhibit Src activity. We hypothesized that one mechanism by which Rack1 facilitates cell-cell adhesion is by interfering with Src-mediated tyrosine phosphorylation of E-Cadherin. This would prevent ubiquitination of E-Cadherin by the E3 ligase Hakai and lysosomal degradation of E-Cadherin. We found that Rack1 attenuates tyrosine phosphorylation of E-Cadherin by Src, disrupts the association of E-Cadherin with Src and Hakai and stabilizes E-Cadherin at cell-cell contacts. Cells expressing wt-Rack1 show a striking increase in E-Cadherin localization at cell-cell contacts compared to cells expressing vector or mutant Rack1 that does not inhibit Src. Furthermore, upon depletion and restoration of extra-cellular calcium, cells expressing wt-Rack1 but not mutant Rack1 form distinct, well-defined E-Cadherin containing cell-cell contacts. Our data supports a crucial role of Rack1 at the Src-E-Cadherin interface in colon cells where Rack1 functions to inhibit Src-mediated E-Cadherin tyrosine phosphorylation and thereby, subverts E-Cadherin from degradation to a recycling pathway.

2061/B440
How to Acquire a Tall and Polarized Epithelial Morphology: The Contribution of Rock-Mediated Contractility.
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Keratinocytes are polarised epithelial cells that have a cuboidal cell shape and distinct basolateral and apical domains. They contain tight Ca2+-dependent contacts with neighbouring cells via E-cadherin receptors. Cadherin-dependent cell-cell contact assembly triggers extensive changes in the actin cytoskeleton organization that culminate in the formation of a cuboidal, polarized cell shape in epithelial cells (morphological polarization). However, the molecular mechanisms responsible for the polarization-specific remodelling of the cytoskeleton are poorly understood. Our previous work has found that, upon induction of cell-cell contacts, thin bundles of actin form
along the periphery of keratinocytes and compact towards the junctions. This is accompanied with increased phosphorylation of MLC in the bundles, suggesting activation of the contractile machinery by cell-cell contacts. Furthermore, cytoskeletal tension and contractility are important for maintaining the integrity of epithelial sheets. As adhesive cadherin receptors activate the small GTPase RhoA, we hypothesise that RhoA-mediated contractility via its effector ROCK is necessary for morphological polarization in epithelial cells. We found that inhibition of ROCK kinase activity by Y27632 compound or depletion of ROCKI, ROCKII or combination of both isoforms did not prevent cadherin-dependent adhesion, but rather induced a 50% drop in the lateral height of keratinocytes. Interestingly, compaction of thin bundles towards cell-cell contacts was also compromised in the absence of ROCKI and ROCKII. These results suggest that without ROCK activity, keratinocytes are unable to fully polarize. These events occurred concomitantly with a reduction of basal levels of phosphorylation of MRLC (pMRLCS19) and MYPT (pMYPTT696 and pMYPTT850) following ROCKI or ROCKII RNAi. Cell-cell adhesion-dependent increase in phosphorylation of the contractile machinery was also perturbed in the absence both ROCKI and ROCKII. In addition to the above defects, we also investigated the participation of ROCK proteins in junction biogenesis and distribution of E-cadherin and actin along the lateral domain of polarizing keratinocytes.

2062/B441
Evidence from In Vivo Cortactin (CTTN) Knock-Down That Podosome-Like Tubulobulbar Complexes Are Involved with Sperm Release.
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Tubulobulbar complexes are actin-related double membrane projections that form at intercellular junctions in the seminiferous epithelium of the mammalian testis. These structures resemble podosomes that form at cell/matrix attachments in other systems and possess a similar actin network containing N-WASP, Arp2/3, cortactin and dynamin. Tubulobulbar complexes are proposed to internalize intact junctions during epithelial remodeling events such as sperm release. If this is true, then interfering with the formation or function of tubulobulbar complexes should delay or even prevent sperm release. In this study, we used an In Vivo siRNA strategy to target cortactin. In SD strain rats, one testis was surgically injected with cortactin RNAi reagents whereas the other testis was injected with non-targeting control reagents. After three days, and in each experiment, the testes of two animals were perfusion fixed for electron microscopy, those of another two animals were perfusion fixed and cryo-sectioned for immunofluorescence analysis, and those of a third pair were processed for immunoblotting. on immunoblots, cortactin was knocked down in experimental testes when compared with levels in control testes. In cortactin RNAi-treated testes, cortactin containing tubulobulbar complexes were present in association with late spermatids in stage VII sections of seminiferous epithelium; however, the complexes were remarkably shorter than in control testes and the bulbar ends of the structures appeared abnormal. Significantly, some mature spermatids remained attached to the epithelium in late Stage VIII with some of these spermatids positioned deep within the epithelium. Adhesion complexes (ectoplasmic specializations) remained associated with some of these spermatids, particularly those near the apex of the epithelium. Our results are consistent with the hypothesis that tubulobulbar complexes internalize intercellular junctions and are part of the sperm release mechanism. Supported by an NSERC Discovery grant to AWV.

Gene Structure and Expression (2063 – 2086)

2063/B442
Low Power Infrared Laser on the Action of a Reducing Agent on Escherichia Coli Cultures.
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Applications of low power laser to treat muscular inflammatory processes have increased worldwide due its photothermal effects on tissues. However, data about the adverse effects of laser on DNA are scarce. The aim of this work was to evaluate effects of low power infrared laser on bacterial cultures proficient and deficient on the repair mechanisms of DNA lesions. E. coli AB1157 (wild type), AB1886 (uvrA-), BH20 (fpg-) e BW9091 (xth-) cultures, in exponential growth phase, were irradiated with infrared laser (830nm) at continuous and pulsed (2.5; 250 and 2500Hz) and at different fluencies (1, 4 and 8 J/cm²). After that, aliquot were spread onto Petri dishes with nutritive medium and incubated (37°C, 18 hours). Colony units forming were counted and survival fractions (SF) were determined. Data obtained of SF, at the higher fluency, for AB1157 were (X and SD): 1.34 SD 0.59 (continuous); 1.21 SD 0.49 (2.5Hz); 1.32 SD 0.31 (250Hz); 1.37 SD 0.74 (250Hz); 1.37 SD 0.75 (2500Hz); for AB1886: 0.73 SD 0.17 (continuous); 0.87 SD 0.31 (2.5Hz); 0.53 SD 0.19 (250Hz); 0.41 SD 0.27 (2500Hz); for BH20: 1.31 SD 0.38 (continuous); 1.02 SD 0.42 (2.5Hz); 1.27 SD 0.25 (250Hz); 1.44 SD 0.54 (2500Hz); for BW9091: 1.06 SD 0.27 (continuous); 1.07 SD 0.15 (2.5Hz); 1.22 SD 0.12 (250Hz); 1.34 SD 0.20 (2500Hz). These data indicate decreasing of survival of E. coli AB1886 cultures exposed to laser radiation at continuous and pulsed modes, at evaluated frequencies. Results obtained suggest that exposition at low power infrared laser (830nm) could induce lesions in the DNA of E. coli cells whose repair may depend of the gene uvrA- product.

\[2064/B443\]

Loops Are Not Twisted.

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By screening all the ligand binding sites in the Protein Data Bank, we have found that while it is geometrically possible that a loop, formed from a protein-chain with residues ZYX would "impersonate" another chain-loop with residues XYZ by a simple twisting of either the loop or the bound ligand, it almost never happens. This fact is rather surprising, since (i) loops in the folded proteins sometimes can be flexible enough to be twisted, but (ii) ligands are almost always extremely mobile before binding to the protein, therefore they can turn around and bind to residue-sequence ZYX as well. In the PDB we identified more than 25,000 protein-ligand pairs (Szabadka, Z., Grolmusz, V.: Proceedings of the 28th IEEE EMBS Annual International Conference, New York City, Aug. 30-Sept 3, 2006., pp. 5755-5758). However, these pairs may contain numerous redundancies: the same polypeptide sequence may be present even in more than 160 PDB entries. After filtering out redundancies in the dataset, we were left with 19,581 distinct binding sites on protein surfaces. For each binding site those residues were collected that were closer to any ligand atoms than 1.05 times the sum of the Van der Waals radii of the two atoms involved. Next we identified the residues containing these atoms: for every binding site an ordered sequence of the residues were created. Then we identified these residues in the amino-acid sequence of the chain of the protein, residues not present in the given binding site were simply substituted by a `--` mark. As the next step, we counted the number of appearances of these subsequences and also their inverted ones in binding sites. For example, we counted the frequencies of both XYZ and ZYX subsequences, and compared the results. We have found that almost never happens that a residue-sequence of XYZ and its inverse, ZYX both bind the same ligand: only 8 such cases occur.

\[2065/B444\]


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The Protein Structure Initiative Material Repository (PSI-MR; http://psimr.asu.edu) provides centralized storage and distribution of information and samples for the 80,000 protein expression plasmids created by PSI researchers. These plasmids are an invaluable resource that allows the research community to dissect the biological function of proteins whose structures have been
identified by the PSI. Researchers can search for and request plasmids from the PSI collection through the repository’s distribution website, DNASU (http://dnasu.asu.edu). Each PSI plasmid is linked to the PSI Structural Genomic Knowledgebase (PSI-SGKB; http://kb.psi-structuralgenomics.org/), which facilitates cross-referencing of a particular plasmid to protein annotations and experimental data. Thus far over 25,000 PSI plasmids are in the process of full-length sequence validation and annotation at the MR, and nearly 16,000 are already available from DNASU. In addition to distributing materials, the MR has sought to simplify the MTA process in order to decrease the time it takes for institutions to deposit or receive plasmids. To achieve this goal, the MR pioneered two documents, the depositor’s agreement, which sets forth the terms enabling the MR to distribute deposited plasmids from outside institutions, and the expedited process MTA, which eliminates the need for researchers to wait for their institutions to sign an MTA. In the future, the MR will continue to make PSI plasmids and data available to researchers and will expand its expedited MTA network so that researchers can receive PSI plasmids without delay.

2066/B445
Single-Molecule Optical-Tweezers Studies of Ribosome Helicase Activity.
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The genetic code in an mRNA is translated by ribosomes into a protein; three nucleotides—one codon—represent one amino acid. Because natural mRNAs contain base-paired regions, the ribosomes need to unwind these structures into single-strands before the mRNA can be translated, thus ribosomes are helicases. We used optical tweezers to apply force to the ends of an mRNA hairpin being translated by a single ribosome. at constant force, the mRNA end-to-end distance increases as the ribosome translates the message and converts double-stranded RNA into a single strand. This technique allowed us to see trajectories of pause-translocation-pause steps corresponding to codon-by-codon translation. The rate of translation is measured as the reciprocal of the mean of the dwell times at each codon. We found that the translation rate increases quickly as force is increased within a narrow force range but the rate plateaus below and above this force range. We also found that the ribosome translation rate on duplex mRNA (low force) is ~50% of the rate on single-stranded mRNA (high force). The observed translation rate dependence on force is inconsistent with a passive unwinding model or the active unwinding models that have been applied to T7 and NS3 helicases. Instead, the data can be fit well by a simple active unwinding model where the ribosome takes full advantage of the thermal breathing of the mRNA for translocation, but it also has active helicase activity for the fraction of time that the junction remains closed. This result suggests that there might be fundamental differences in the unwinding mechanism of ribosomes and other helicases. Furthermore, we observed that the translocation lifetimes have a single exponential distribution and show no dependence on force, or on the concentrations of elongation factors EF-Tu and EF-G. Both this observation and the modeling of ribosome helicase activity suggest that the movement of a ribosome on mRNA by one codon is a single-step process with no sub-steps. Our results show a tight coupling of the ribosome translation and helicase activities, and should facilitate the understanding of translation regulation mechanisms, such as frameshifting.

2067/B446
Transcriptional Changes in [PSI+] Strains of Saccharomyces cerevisiae.
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Prion proteins are involved in neurodegenerative diseases. Yeast provides a valuable model system to study prion proteins' effects on cells. We investigated transcriptional changes in yeast
strains containing the prion conformer of the Sup35 gene protein which codes for a translational release factor protein (eRF3). The prion conformer's cellular presence will result in altered translation termination and the strain is designated as [PSI+]. We conducted transcriptional profiling using DNA microarrays on isogenic [PSI+] and [psi-] yeast strains (strain L1763 and strain 74D). The results were that there are a lot of small, but statistically reproducible, changes in retrotransposon genes' expression in [PSI+] when compared to [psi-]. Changes in retrotransposon RNA are involved in genomic changes and can give rise to genetic variation. Previous work done by others has suggested that [PSI+] generates different phenotypes in yeast with different genetic backgrounds. We investigated whether the same changes in levels of retrotransposon and other RNAs would be found when we compared gene transcription in these different strains. When strains 74D and L1763 were compared, we found that about 3-4% of the genes are altered in their expression in both strains in [PSI+]. Of these genes, about a quarter (25%) are underexpressed or overexpressed in both the 74D and L1763 yeast strains. The remaining genes differ or change in both strains, but the changes are in different directions. The data reveal that the presence of Sup35 protein in its prion conformation causes many subtle, but statistically significant changes in the levels of mRNAs in cells. The genetic background of the strain seems to be important as some changes are found regardless of it while other changes vary depending on it. for example, in both genetic backgrounds we found changes in the mRNA levels of retrotransposon genes, but some gene expression differences were only found in one strain. Our studies may help to explain the prior finding that [PSI+] generates different phenotypes in different genetic backgrounds. This may be because there is differential gene expression in these [PSI+] yeast strains.

2068/B447
Role of 5'UTR and 3'UTR Variants of the SftpA1 and SftpA2 Genes in the Regulation of Translation.
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Surfactant protein a (SP-A) is produced by pulmonary alveolar epithelial type II cells, and plays an important role in host defense and surfactant-related functions. Human SP-A is encoded by two functional genes (SftpA1, SftpA2). Structural and functional differences, as well as splice and sequence differences have been observed at the 5' untranslated region (UTR) and 3'UTR, respectively, between SftpA1 (SP-A1) and SftpA2 (SP-A2). Here, we investigated In Vitro the differential role of 5'UTR splice and 3'UTR sequence variants on translation. We studied the impact on translation of two SP-A1 5'UTR variants (AD, AD') and two SP-A2 5'UTR variants (ABD, ABD') alone or in combination with other structural mRNA elements known to control translation: poly(A) tail and 3'UTR. We generated constructs (n=17) containing the Luciferase reporter gene flanked by one of the SP-A 5'UTR splice variants and one of the SP-A 3'UTR sequence variants (SP-A2: 1A0, 1A3 and SP-A1: 6A2, 6A4). mRNA transcripts were prepared from each construct with and without poly(A). In Vitro translation was performed using a Rabbit Reticulocyte Lysate System (Promega®) in the presence of [35S]-Methionine. Luciferase activity was determined by the Dual-Luciferase® Reporter Assay System (Promega®) and protein expression was analyzed by SDS-PAGE and fluorography. We found: 1) transcripts containing SP-A2 5'UTR (ABD, ABD') variants were translated more efficiently than SP-A1 variants or no SP-A 5'UTR; 2) in SP-A1 5'UTR and no 5'UTR containing transcripts, the addition of a 3'UTR increased translation; but no significant differences were found among the 3'UTR variants tested; 3) the poly(A) increased translation in the absence of SP-A 3'UTR; 4) SP-A2 5'UTRs and poly(A) showed an additive effect on translation when 3'UTR was omitted. Conclusions: 1) sequences located at the SP-A2 5'UTR (exon B) act as positive regulators of translation in an additive manner with poly(A); and the 3'UTR abrogates this additive effect; 2) the 3'UTR affects the poly(A)-mediated translation enhancement in all transcripts. In Vivo experiments are needed to
further gain insight into the complexities of the role of these mRNA elements on translation.
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2069/B448
Differential Expression of NANOG and Its Pseudogene NANOGP8 in Differentiated Cells.
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The homeodomain gene NANOG is a transcription factor and has been identified as one of the
factors essential for maintaining pluripotency in embryonic stem cells. It has been widely
accepted that NANOG is not expressed in differentiated cells. We recently reported the up-
regulation of pluripotency specific genes OCT4, NANOG, SOX2 and REX1 in human fibroblasts
by manipulating culture conditions. We followed that study by investigating NANOG expression in
eight different human cell types and one tissue (heart). Using RT-PCR and qRT-PCR we
detected expression of NANOG transcripts in all the cell types and heart tissue. These transcripts
were further analyzed to determine whether they represented the parental NANOG or one of its
pseudogenes NANOGP8 since these two sequences share about 99.5% homology in their open
reading frames (ORF) and therefore difficult to distinguish by PCR. Analysis of nucleotide
sequence revealed that a Sma I restriction site (CCCGGG) present in 3' untranslated region
(UTR) of parent NANOG was absent in NANOGP8 (GCCGGG) due to a C to G transversion. A
2031 bp RNA that included the NANOG ORF and most of its 3' UTR was amplified and digested
using Sma I enzyme. Neonatal fibroblasts, HeLa cells, SH-SY5Y neuroblastoma cells,
mesenchymal stem cells and embryonic stem cells transcribed parent NANOG exclusively, while
smooth muscle cells expressed only NANOGP8. Adult fibroblasts, human umbilical vein
endothelial cells and heart tissue expressed both forms. Western blot analysis and
immunocytochemistry using a monoclonal antibody against NANOG confirmed that
NANOG/NANOGP8 protein was expressed in all the cell types. Our observations indicate that
expression of NANOG may not be restricted exclusively to embryonic stem cells but rather
expressed in a wide variety of cells/ tissues. We hypothesize that NANOGP8 has the potential to
substitute for NANOG as a transcriptional activator in cells that do not express parent NANOG.

2070/B449
uChIP Analysis of Histone Modifications H3K4m3, H3K9m2, and H4k16ac of NANOG,
POU5F1, and SOX2 in Bovine Blastocysts Following Somatic Cell Nuclear Transfer.
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The modification of histones is an epigenetic mechanism used to regulate gene expression. Abnormal modifications of histones have been proposed to contribute to the reduced
development to term and low success rate of embryos following somatic cell nuclear transfer
(scNT). The inability to reprogram epigenetic markers such as histone modifications found in the
fibroblast donor cell line, especially in histones associated with establishing totipotency, could add
to the reprogramming deficiencies associated with scNT. Two histone modifications associated
with transcriptional activation; H3K4m3 and H4K16ac, and one histone modification associated
with repressing transcription; H3K9m2, were considered with their association to three genes
known to contribute to maintaining totipotency: NANOG, POU5F1, and SOX2. The uChIP
protocol was performed by using antibodies specific for each histone modification, followed by real
time PCR (qPCR) analysis in order to compare bovine cumulus cells, scNT blastocysts, and In
Vitro fertilized (IVF) blastocysts. The gene POU5F1 was not highly associated with any of the
histone modifications regardless of the treatment group. The Sox2 gene showed high levels of
association with all three histone modifications in both IVF and scNT blastocysts, but very
minimal association with the modifications in cumulus cells. The gene Nanog was highly
associated with all three histone modifications in the scNT embryos, while only highly associated
with the modifications H3K4m3 and H3K9m2 in IVF embryos. These data indicate that aberrant reprogramming of histone modifications in bovine scNT does occur and could contribute to the low success rate of scNT embryos.

2071/B450

Efficient Non-Viral Gene Delivery by Biodegradable Nanoparticles in Mouse Pluripotent Stem Cells.

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Gene delivery to pluripotent stem cells offers great promise for tissue regeneration and delivery of therapeutic proteins. However, one major barrier that stem cell researchers face is a lack of quick, inexpensive, and efficient delivery methods. While virus-based systems and nucleofection are capable of providing the high efficiency often desired, they are constrained by the amount of time and labor required for either virus production (virus-based systems) or the optimization of delivery conditions for each cell line (nucleofection). Not surprisingly, non-viral gene delivery systems are quickly gaining recognition as being faster and easier alternatives to methods involving viral systems or nucleofection. Many cationic lipids have been studied both In Vitro and In Vivo for gene delivery purposes. However, the cationic lipid transfection reagents commonly used to transfect primary cells and embryonic stem cells often result in low transfection efficiency and poor cell viability. Here, we report an enhanced non-viral gene delivery technology (based on novel, biodegradable, polymeric compounds) that provides high transfection efficiencies and low cytotoxicity in both mouse embryonic fibroblast (MEF)-dependent and MEF-independent mouse embryonic stem (mES) cell lines. Even in the presence of 15% serum, these biodegradable nanoparticles exhibit significantly higher transfection efficiencies in ES-D3 (80 ± 5%) and ES-E14Tg2a (80 ± 5%) mES cell lines than do other commonly available lipid-based transfection reagents. Furthermore, cell viability is high (typically 75-90%) in both cell lines. Importantly, these biodegradable nanoparticles have minimal toxicity and do not adversely affect mES cell colony morphology or cause nonspecific differentiation.

2072/B451

Human GW182 Protein Contains Divergent Functional Domains in Regulating Translational Repression.

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MicroRNA mediated post-transcriptional regulation of gene expression have become a major focus in many biological processes. GW182 is a marker protein of GW bodies (GWB, also known as mammalian P-bodies) and binds to Ago proteins which are core components in the RNAi induced silencing complex. GW182 is critical for the formation of GWBs and miRNA-mediated silencing. Translational repression of tethered Ago2 to 3'UTR requires GW182 and tethered GW182 exerts a stronger repression than tethered Ago2. The goal for this study is mapping the repression domain(s) in GW182 and dissecting the molecular event of translational repression. Two different Renilla luciferase reporters with the dual luciferase system were used. The first reporter contains a 5BoxB RNA structure to tether N-terminal λN-hemagglutinin (NHA) tagged proteins to the 3'UTR. The second reporter RL-20bg contains 7 miR-20 target sites which forms bulge (bg) structures with miR-20. GW182 contains several glycine/tryptophan-rich (GW-rich) regions, a glutamine/asparagine-rich (Q/N-rich) domain, and a C-terminal RNA recognition motif (RRM). The middle region (aa896-1219) containing Ago hook domain and C-terminal region (aa1670-1962) containing RRM were identified to induce silencing using tethering assays. Finer mapping showed that the C-terminal RRM was important for the tethering repression but not Ago hook in the middle region. Knocking down endogenous factors in miRNA silencing pathway such as GW182, RCK/p54, or TNRC6B did not affect the repression triggered by these two silencing domains. Most importantly, overexpressed aa896-1219 and its C-terminal deletion construct aa896-1045 (minus the Ago hook), interfered with the miR-20 reporter function. Thus the Ago Hook domain did not play a role in repression. Collectively, these findings shed light on functional
preferences of different regions in GW182 and provided evidence that these functional domains could work co-operatively in miRNA-Ago induced translational silencing.

2073/B452
GSK and SCF-Mediated Regulation of the Stability, Localisation and Function of the Transmembrane Transcription Factor CREB-H.
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CREB H is a member of the specialised set of ER-localised transmembrane transcription factors that regulate adaptive responses to ER-associated stress. Their activity is controlled by ER retention via association with specific sensors which recognise specific forms of ER stress. Stress-induced release from ER-retention and forward transport to the Golgi, results in cleavage by Golgi-proteases, to produce the N-terminal active form which travels to and effects adaptive transcriptional responses in the nucleus. In the case of CREB-H, pro-inflammatory cytokines such as IL6 and TNFα have been reported to elicit CREB-H cleavage, resulting in up-regulation of certain acute-phase response genes, although the mechanisms are unknown. Here we show that CREB-H localisation and cleavage can also be regulated by insulin. We identified a conserved motif in the N-terminus of CREB H, with homology to GSK target motifs and examine targeting and degradation by the SCFβTrCP E3 ubiquitin ligase. We demonstrate that substitution of conserved serine residues has a stabilising effect on the protein, with a significant stabilisation of the nuclear processed form. We demonstrate that expression of a dominant negative Cul1 construct specifically enhances expression of the CREB H, but not the mutant form; similarly the co-expression of βTrCP further modulated the steady state levels, consistent with the E3 ligase hypothesis. We demonstrate that the stable mutant has both retained and enhanced transcriptional activity, and accumulates to a greater level in response to physiological cleavage stimuli. Thus CREB-H may integrate different forms of stress signals at the ER and we suggest a model where, in addition to regulation of transport and cleavage, regulation of rapid turnover can modulate the extent and duration of transcriptional activation for different forms or combinations of stress at the ER.

2074/B453
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Plant LIM proteins and the related animal Cystein-Rich Proteins (CRPs) localize to both the nuclear and cytoplasmic compartment. The animal CRPs were shown to be implicated in the organization of the actin cytoskeleton and in the integration of stress signals. A key role in actin filament bundling and actin cytoskeleton stabilization was also demonstrated for plant LIM proteins (Thomas et al., 2006; 2007). We have been investigating the nuclear functions of the tobacco protein NtWLIM2. Similar to the other plant LIM proteins, NtWLIM2 shows a dual nucleo-cytoplasmic localization. Its coding sequence was isolated during a screen for Arabidopsis histone H4 promoter-binding proteins (Shen and Gigot, 1995) suggesting it has DNA binding properties. More recently, binding of the protein to a cis-element of the Arabidopsis promoter was experimentally confirmed using electrophoretic mobility shift assays. Interestingly, each of the two LIM domains of the protein was found to contribute to this binding (S. Gatti, PhD thesis). In order to check if this binding can activate the expression of a gene in Vivo we used a transactivation system comprising an effector p35S:NtWLIM2 and a promoter-reporter (pH4:LUC) plasmid in transiently transformed Arabidopsis protoplasts. We found that increasing amounts of NtWLIM2 plasmid DNA led to an increase of histone H4 promoter-driven reporter gene activation when compared to the luciferase activity registered in presence of the same amounts of a control
plasmid. However, the same experiment performed with a luciferase construct driven by four copies of the cis-element fused to a 35S-core promoter yielded no increase of the basic reporter activity, suggesting that NtWLIM2 activation involves a second cis-element with possibly a corresponding ligand protein. This research was supported by a grant and a PhD (SG) and post-doctoral (DM) fellowship from the Luxembourg Ministry of Culture, Higher Education and Research.

2075/B454

**Differential Regulation of SEPT9 Transcripts by p53.**

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The septins are a family of 14 genes thought to function primarily as scaffolds which recruit other proteins to particular cellular locations. The complexity of SEPT9 genomics and resultant transcripts has led us to consider the regulation of this gene. Bioinformatic analysis revealed many potential transcription factor binding sites including numerous putative p53 responsive elements within a 3kb region upstream of the transcription start site of SEPT9_v4* while such sites are infrequent proximal to other SEPT9 transcripts. This transcript can be translated by cap independent mechanisms and is one of two transcripts that encode the SEPT9_i4 isoform (see Hum Mol Gen 2007;16:742-752). We hypothesised that some, but not all, SEPT9 transcripts would be stress responsive via a p53 dependant pathway. We used a range of cells with wild type p53 and an isogenic pair of lines with and without p53 to investigate the cellular physiology of SEPT9 after genotoxic and non genotoxic stress. A range of cellular stresses including DNA damage induce p53 protein expression and concomitant induction of SEPT9_v4* mRNA and SEPT9_i4. In contrast, the levels of other SEPT9 transcripts and proteins show no significant change. In isogenic p53 null cells there is no induction of SEPT9_v4* mRNA or SEPT9_i4. Reconstitution of the p53 pathway in p53 null cells restores induction of SEPT9_i4 protein under conditions of cellular stress. Chromatin immunoprecipitation experiments have confirmed that the bioinformatically observed p53 responsive elements do indeed bind p53 protein. Furthermore DNA fragments containing these elements are capable of driving transcription of a reporter gene in a p53 dependent manner. Taken together these data indicate that there are differential responses of SEPT9 transcripts to cellular stress mediated, at least in part, by the p53 pathway.

2076/B455

**Differential Regulation of Th1 and Th2 Chemokines Expression by Superoxide Dismutase in TNF-α/IFN-γ Stimulated HaCat Cells.**

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Exposure of the keratinocytes to interferon-γ (IFN-γ) and tumor necrosis factor alpha (TNF-α) leads to abnormal expression of chemokines. Dysregulated expression of chemokines increase infiltration of monocytes/T cells into the site of inflammation in the skin. Th1 chemokines (CXCL9, 10, 11) and Th2 chemokines (CCL17, CCL22) are considered to be pivotal mediators in the inflammatory responses during the development of atopic dermatitis. This study was performed to determine the regulatory function of superoxide dismutase (SOD) on the TNF-α/IFN-γ-induced expression of Th1 and Th2 chemokines in the human keratinocyte cell line HaCaT. Treatment of HaCaT cells with a cell-permeable SOD, Tat-SOD led to decrease in TNF-α/IFN-γ-induced ROS generation. Pretreatment with Tat-SOD significantly inhibited TNF-α/IFN-γ-induced expression of Th2 chemokines CCL17 and CCL22 but had no effect on the expression of Th1 chemokines (CXCL9, 10, 11) in HaCaT cells. Tat-SOD inhibited TNF-α/IFN-γ-induced NF-kB DNA binding activity, IkBα degradation and activation of mitogen-activated protein kinases (MAPK) but had no effect on the STAT activation. These data suggest that SOD differentially regulate expression of Th1 and Th2 chemokines by inhibiting activation of NF-kB as well as MAPK in TNF-α/IFN-γ stimulated HaCaT cells.
2077/B456  
The Vcsa1 Gene Affects Erectile Dysfunction in Spontaneous Diabetic Rats.  
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The association of type 2 diabetes and microvascular complications can be explained by endothelial dysfunction in the pancreatic microvasculature. It causes a subtle islet ischemia and subsequent beta cell damage. Vcsa1 gene encoding Sialorphin plays important roles in erectile physiology. Sialorphin belongs to a family of peptides which act as endogenous neutral endopeptidase (NEP). This protein has been demonstrated to be involved in many physiological processes such as pain perception, sexual behavior, and erectile function. Testosterone has regulated central and peripheral erectile function induces Vcsa1 expression. Type 2 diabetes mellitus (T2DM) is a major risk factor for erectile dysfunction (ED). However, there was no significant difference of testosterone level between spontaneous diabetic rat and non-diabetic rat. Therefore we asked whether Vcsa1 gene normally expressed in Type 2 diabetes mellitus with ED. The aim of the present study is to investigate that the Vcsa1 gene expression affects erectile dysfunction. To detect Vcsa1 gene expression, we used spontaneous diabetic rat with ED and normal rat without ED as a control of spontaneous diabetic rat and sampled corporal smooth muscle (CSM) from both groups. Quantitative RT-PCR was used to compare Vcsa1 gene expression level in corporal smooth muscle. The result showed significant decrease of Vcsa1 gene expression was noted in spontaneous diabetic rats than normal rats. These results suggest that Vcsa1 gene can act as markers of ED in Type 2 diabetes and may be one cause of ED.

2078/B457  
P62/SQSTM1 Binds Directly to Keap1 to Induce Nrf2.  
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Oxidative stress represents an over-production of reactive oxygen species in a cell relative to its ability to detoxify such species, and this can lead to cellular damage. The Keap1-Nrf2 signaling pathway can be activated by oxidative stress and the transcription factor Nrf2 up-regulates target genes through a DNA regulatory sequence called the antioxidant response element (ARE). Genes encoding classical phase II enzymes, antioxidant proteins and anti-inflammatory proteins are induced by Nrf2. In addition, Nrf2 regulates proteins that repair protein damage. The p62/SQSTM1 autophagy-related protein is a member of the protein battery induced by Nrf2 in response to oxidative stress, and induction of p62 is severely inhibited in cells from Nrf2 knock out mice. Under basal conditions, Nrf2 is rapidly degraded and the BTB-Kelch protein Keap1, which uses its C-terminal propeller-like Kelch domain to interact directly with the Neh2 domain of Nrf2, controls this. The binding between Nrf2 and Keap1 is believed to occur in the cytoplasm. Keap1 is an adaptor protein for the Cul3 ubiquitin ligase complex and upon binding to Keap1, Nrf2 is ubiquitinated and degraded by the proteasome. Recent studies have suggested that p62 may contribute to the induction of the Keap1-Nrf2 pathway, but the mechanism is not known. We show that p62 binds directly to the Kelch domain of Keap1, thereby inhibiting the interaction between Keap1 and Nrf2. We show that p62 contains a short sequence motif just C-terminal to the recently identified LC3-interacting region (LIR) that resembles the ETGE motif utilized by Nrf2 for its interaction with Keap1. This motif therefore mediates the interaction between p62 and Keap1, and this motif was found to be essential for the ability of p62 to induce ARE-Luciferase gene reporter assays.

2079/B458  
Genetic Association of Diabetic Candidate Genes (TCF7L2, SLC30A8, HHEX, CDKAL1, CDKN2A B) with Type 2 Diabetes in Spontaneous Rat Model.

- 1003 -  
2009 ASCB Regular Abstracts
Type 2 Diabetes is a multiple complex disease characterized by chronic hyperglycemia caused by the destruction of the balance between pancreatic islet function and peripheral insulin sensitivity, and its incidence caused by the interactions of various genetic and environmental factors. By this time, few genes are found through the candidate gene approach have been confirmed to be correlated with type 2 diabetes. (e.g. PPAR G, KCNJ11, CAPN10, and TCF7L2) There’s identified several novel genes (SLC30A8, HHEX, CDKAL1, CDKN2A/CDKN2B, IGF2BP2, FTO) which associated with type 2 diabetes. Particularly, the more genetic variations augment the more incidences of diabetes increases. We wonder whether the Novel genes can be also applied to spontaneous rat models. To confirm this inference, in this study we verified the association of those genes with type 2 diabetes in spontaneous rat models. And we analyzed whether genetic variations would differ according to strains and sex or not. Two experimental populations were considered: SD (Normal), DM (Diabetes Mellitus) and each group are divided into two groups by sex. Before PCR, we selected five novel genes (SLC30A8, HHEX, CDKAL1, CDKN2A/CDKN2B) among those seven genes which are highly correlated to type 2 diabetes.

And then we performed PCR method using type-specific primers which are contained from those genes and Electrophoresis. In our study we identified genes that significantly correlated with type 2 diabetes in spontaneous rat. Because these type 2 diabetic characteristics in our rat models occurred spontaneously and are inherited to a descendant, these rat models have many advantages by comparison with chemical induced diabetes rat model. Also if transgenic rat model is made by using these genes which is verified from this spontaneous diabetes rat, it is very useful in type 2 diabetes treatment and study as a human type 2 diabetes animal model.

2080/B459
Analysis of Variations in the 3-UTR of Cyclooxygenase-2 in Cancer.
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Overexpression of Cyclooxygenase-2 (COX-2) is seen in several different types of cancer, including prostate and colon and it has being related to increased cell replication, metastatic capacity and neovascularization; still, the reason for such increase in production is not yet clear. It has been recently reported that the length of the 3-UTR shifts depending on the proliferation of the cell line, thus changing the capacity of miRNA to downregulate gene expression. To understand this phenomenon in a specific context we have studied the COX-2 3-UTR of different cell lines under different conditions related to stimulation of cell proliferation and stimulation of COX-2 expression then looking for changes in size and sequencing the products in order to look for missing target sites for miRNA control compared to a wild COX-2 3-UTR.

2081/B460
Heterologous Expression of the Chimeric Sag1/2 of Toxoplasma gondii in the Yeast Pichia pastoris.
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Toxoplasma gondii, the etiological agent of toxoplasmosis, is widespread throughout the world (Bhopale, 2003). Routine diagnosis of toxoplasmosis is based on serological detection of antibody in the patient. Attempts to produce antigens through safer means, such as recombinant DNA technology, have been made. Progress has been focused on the surface antigens, such as SAG1 and SAG2, which are the major surface antigens of tachyzoites (Couvreur et al., 1988). Chimeric SAG1/2 expressed in Escherichia coli has been shown to produce partial protection against a lethal infection of T. gondii (Yang et al., 2003). This is likely due to the incorrect folding of the recombinant protein when produced in the prokaryotic cells (Mishima et al., 2001). This
study seeks to clone and express the chimeric SAG1/2 gene in *Pichia pastoris*, which possess ability to produce recombinant proteins with conformation that is almost similar to the native proteins. The gene was cloned into the pPICZα a expression vector, under the AOX1 promoter. The antigen (rSAG1/2) was expressed along with the prepro sequence so that it could be excreted out of the *P. pastoris* cells. Upon SDS-PAGE analysis it was found that the rSAG1/2 was best expressed after 72 hours of induction and the optimum concentration of methanol for induction was 1%. The rSAG1/2 was expressed with an intact polyhistidine tag, which could be used for purification. Eighty human serum samples, including 60 from confirmed cases of toxoplasmosis, were tested against the purified rSAG1/2 in western blots. Results showed that the rSAG1/2 reacted with 57 sera from the toxoplasmosis cases but none with the *Toxoplasma*-negative serum samples. The results thus indicate that the recombinant SAG1/2 was specific for *Toxoplasma* antibody. The capability of the rSAG1/2 to induce protective immunity in BALB/c mice will be followed.

**2082/B461**

**Mediation of Immunoglobulin Gene Transcription and Diversification by the Histone Deacetylases HDAC1 and HDAC2 in the Chicken DT40 B-Cell Line.**

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The adaptive immune system of vertebrates requires an enormous variety of antibody to protect the organism from the myriad of foreign antigens. This diversity is acquired by a process of DNA sequence diversification at the immunoglobulin (Ig) gene locus. In chicken, this role is mainly played by gene conversion, a type of homologous DNA recombination. Previous works reported that homologous recombination is associated with the local hyper-acetylation of nucleosomal histones. Furthermore, we demonstrated that the treatment of the chicken DT40 B-cell line by a histone deacetylase (HDAC) inhibitor, trichostatin a (TSA), highly stimulates gene conversion. To further investigate the involvement of HDACs in the regulation of Ig gene diversification, we examined the effects of the homozygous deletion of two class I deacetylases, HDAC1-/- and HDAC2-/-.

HDAC2-/- cells showed an increase in the incidence of gene conversion at both the Ig light chain and heavy chain loci, concomitantly with the local increase of chromatin histone acetylation and of Ig mRNA expression. In contrast, HDAC1-/- cells showed an increase of conversion frequency only at the Ig light-chain locus, while transcription and histone acetylation levels remained unchanged for the light-chain and decreased for the heavy-chain in comparison to wild type cells. Taken together, these results suggest that the Ig light- and heavy-chain loci are differentially controlled by HDAC1 and HDAC2, which may be directly or indirectly involved in the regulation of gene conversion, transcription and histone acetylation. As biochemical analysis of individual HDAC and detailed examination of local chromatin modifications are expected to uncover the mechanistic pathways of these regulations, we are currently focusing on the differences between the carboxy-terminal domains of HDAC1 and HDAC2 and on the residue specificity of HDACs in histone deacetylation.

**2083/B462**

**Retention of Specific Intronic Sequences Is an Important Feature of Post-Transcriptional mRNA Regulation in Neuronal Dendrites.**

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Gene expression is regulated by a number of well-characterized processes from transcription to translation. While normally considered as being confined to the nucleus, recent publications have identified retained introns in cytoplasmic mRNA. In both neuronal dendrites and anucleate platelets, retained intronic sequences have been associated with cellular function, proper protein
expression and responsiveness to external stimuli. Here we report that the retention of introns in the mRNA of neuronal dendrites is a general process and may serve as a post-transcriptional mechanism of gene expression regulation for a larger number of transcripts than previously reported. Dendritically localized mRNA with retained introns were detected by microarray, and confirmed for localization by in situ hybridization and next generation sequencing. All candidate sequences were analyzed computationally for common features found in retained introns versus non-retained introns from our screen. Portions of retained intronic sequences were exogenously expressed in neurons and assessed for their intrinsic targeting ability as well as their effect on endogenous transcripts. Our results show that the retention of specific intronic sequences within dendritic mRNA has a role in dendritic localization of transcripts.

2084/B463
Dimerization of CAC-rich RNA Localization Elements Contributes to Their Function In Vivo.
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We have shown previously that CAC-rich RNA localization elements are widespread cis-elements that drive RNA localization in oocytes and neurons (Andken et al., 2007 BMC Bioinformatics 8:274). These regulatory elements are characterized by a statistically high number of short CAC-containing 3-7 nucleotide motifs that are required for localization. While these short motifs are required for localization and compete for RNA localization factors in vivo, they are not sufficient to localize themselves when present as tandem copies in a single transcript. This suggests that the structure of CAC-rich RNA localization elements may also be important for their function. To begin to identify RNA structures within CAC-rich RNA localization elements we searched for secondary structural elements that are conserved between orthologous genes of closely related Xenopus and acidian species, but we found none. We then searched for the potential for CAC-rich RNA localization elements to form intermolecular dimers as does the bicoid localization element in Drosophila. A computational analysis suggested that formation of RNA dimers may be an essential feature of this class of RNA localization elements. To test one molecular dimerization model, we focused on the Xcat-2 mitochondrial cloud CAC-rich RNA localization element (MCLE). Using quantitative RNA localization assays and compensatory mutations, we show that localization of a mutant MCLE lacking the ability to self-dimerize can be rescued by a compensatory mutant in trans that restores intermolecular base pairing. Since dimerization of the MCLE involves over 80 intermolecular base pairs it is likely that multimerization in addition to dimerization of these sequences occurs in vivo, and, in principle, such RNA-RNA interactions could promote the formation of RNA granules. These results broaden our understanding of the sequences that polarize the expression of hundreds, if not thousands, of genes in vertebrate cells and also provide insight into how interactions between multiple transcripts may promote the formation of subcellular structures involved in the localization process.

2085/B464
Analyzing the Effects of mRNA Localization to Microtubules on Protein Expression and Function.
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Subcellular localization of RNA is an important mechanism regulating gene expression, and it is essential in many organisms for controlling cell fate and embryonic development. Correct segregation of RNA molecules requires sequence/structural features within the transcript (“zipcodes”), and trans-acting factors that recognize and bind to these zipcodes. In order to achieve spatial restriction of protein products within a cell, RNA transport is often coupled to translation control mechanisms to ensure that the transcript is not prematurely translated before reaching its destination. Recently, numerous mRNAs have been found localized at the mitotic spindle in Xenopus egg extracts, but the RNA sequences directing this localization remain unknown. Furthermore, the effects of spindle targeting on the expression of these transcripts are
also not known. We are utilizing both conventional mapping approaches as well as high-throughput selection to identify zipcodes within microtubule-localized mRNAs. Of particular interest is the EWS transcript, which encodes a protein with oncogenic potential that is frequently mutated in Ewing’s Sarcoma, and that both binds RNA and acts as a transcription factor. Identification of zipcodes within EWS will allow us to investigate how RNA localization affects the expression and function of EWS protein in *Xenopus* egg extracts.

**2086/B465**

**Analysis of Piwi and Pirnas in Xenopus Oocytes.**

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Small RNAs (si- and miRNAs) control the stability and translational activity of mRNAs in a wide range of organisms. However, the recently discovered piRNAs are a diverse class of small RNA that play an important role in transposon control, and may also have other roles. We used oocytes from the African frogs *Xenopus laevis* and *Xenopus tropicalis* to identify and characterize both piRNAs and Piwi proteins. Two different Piwi proteins are expressed in *Xenopus*, Xiwi and Xili. Xiwi localizes to the Balbiani body in early oocytes, which is the future site of germ cell formation, and to the vegetal hemisphere and meiotic spindles in mature oocytes. Depletion of Xiwi does not affect meiotic spindle assembly in vitro, suggesting that Xiwi and piRNAs are passive cargo on microtubules. Using high-throughput sequencing we found that the vast majority of small RNAs present in *Xenopus* oocytes are piRNAs, with little evidence of miRNAs and endo-siRNAs. We found that piRNAs target both transposons and mRNAs in equal proportions, suggesting that piRNAs may regulate gene expression. Furthermore, using single cell sequencing we found that the expression of piRNAs is conserved between different cells from the same individual, but differs between individuals. We found that *Xenopus* extracts will cleave RNAs complementary to sequenced piRNAs in a Xiwi-dependent manner and that these cleaved RNAs bind to Xiwi. However, we find little evidence of a major role for piRNAs or Xiwi in regulating the translation of mRNAs containing piRNA binding sites, suggesting that piRNAs function in a fundamentally different manner than miRNAs. Our results demonstrate that *Xenopus* is an attractive system to explore the role of piRNAs in a variety of cellular processes, including RNA localization and translational regulation.

**Mechanisms of Nuclear Transportation (2087 – 2097)**

**2087/B466**

**C/EBPβ-Mediated Repression of the Ric-8b Gene Requires the SWI/SNF Activity.**

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Ric-8b regulates G-protein signaling and the alignment of the mitotic spindle during cell division. We have previously described that the Ric-8b gene is negatively regulated by the transcription factor C/EBPβ (LAP*) during osteoblast differentiation. C/EBPβ can interact with histone deacetylases (HDACs) to inhibit gene transcription. Similarly, C/EBPβ can interact with SWI/SNF chromatin remodeling complexes to activate transcription. Therefore, we assessed whether C/EBPβ-HDAC and/or C/EBPβ-SWI/SNF complexes are contributing to Ric-8b expression in osteoblasts. We report that transient over-expression of different HDACs (HDAC3, HDAC4, HDAC8 and HDAC11) does not affect Ric-8b gene promoter activity. Accordingly, chromatin immunoprecipitation (ChIP) analyses demonstrate that Ric-8b repression during osteoblasts differentiation does not involve changes in histone acetylation at the Ric-8b promoter. Alternatively, we observed that transient over-expression of Brg1 or Brm, the catalytic subunits of the SWI/SNF complex, inhibits Ric-8b promoter activity in osteoblastic cells. In contrast, forced expression of a Brm mutant form that assembling inactive SWI/SNF complexes, impairs C/EBPβ
TUESDAY

2088/B467

Over-Expressed Hsecurin Drives Metastasis through Activation of GEF-H1.

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hSecurin, also called PTTG1 (pituitary tumor-transforming gene 1) is over-expressed in several tumor types and especially in invasive tumors. In normal cells it is a mitosis regulator and in tumor cells it functions as an oncogenic transcription factor. How hSecurin drives cancer metastasis is still unclear. One of the possible mechanisms may be attributed to the activation of its target genes involved in metastatic pathway. Using microarray analysis, we demonstrate that ectopically over-expression of hSecurin in hSecurin null HCT116 cells causes an increased expression of Rho guanine nucleotide exchange factor-H1 (GEF-H1). GEF-H1 itself is also an oncogene and its activator, RhoA, can modulate cell migration and polarity by reorganizing the actin cytoskeleton. We have isolated and analyzed the GEF-H1 regulatory sequence (1,698 bp). Using 5’ deletion mutant constructs of GEF-H1 promoter, we showed that the region -1160 to -1140 containing putative hSecurin binding site 1 is critical for the GEF-H1 promoter activation. hSecurin directly binds to this site in EMSA and Chip assay. These results prove that GEF-H1 is one of the direct targets of hSecurin. RNA-interference-mediated knockdown of hSecurin in highly metastatic (MDA-MB-231) cancer cells decreased the expression of GEF-H1. RhoA is also inactivated by reduction of hSecurin, thereby reducing cell adhesion, cell spreading, invasion and motility in MDA-MB-231 breast carcinoma cells, without affecting proliferation (or inducing apoptosis). Conversely, ectopic hSecurin expression in non-metastatic (MCF-7) cancer cells led to gene expression patterns of GEF-H1 increased. Hence, the level of GEF-H1 expression is significantly correlated with that of hSecurin expression by hSecurin knockdown or over-expressed approaches. Xenografts of hSecurin-depleted tumor cells showed these tumor cells proliferated normally. Preliminary metastatic experiments indicated that tumor cells over-expressed of hSecurin drive metastasis in vivo. However, depleted of hSecurin reduce metastatic potentials. Our results provide evidences that one of the mechanisms over-expressed hSecurin promotes metastasis is through the activation of GEF-H1-RhoA pathway.

2089/B468

The Role of the Mediator Tail Domain in Transcription and Drug Resistance.

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We have found previously that resistance of budding yeast cells to microtubule-destructilizing drug benomyl is increased by deleting SIN4 gene. SIN4 deletion does not improve benomyl resistance by increasing stability of microtubules but by elevating expression of a plasma membrane transporter Flr1p that mediates resistance to benomyl. The aim of this work was to determine the mechanism of how sin4Δ mutation increases expression of the FLR1 gene and whether sin4Δ mutation increases resistance only to benomyl or also other drugs. Sin4p is a component of the Mediator complex of RNA polymerase II and mediates attachment of the tail domain to the middle module of the Mediator. In the absence of Sin4p, the tail domain composed of Med2p, Med3p, and Gal11p subunits is physically detached from the rest of the Mediator and functions independently. Our chromatin immunoprecipitation results indicate that in sin4Δ cells the free tail domain behaves as a promiscuous transcriptional co-activator that increases transcription by
facilitating recruitment of other transcriptional co-activators and assembly of the preinitiation complex at diverse promoters. In agreement with these results, deletion of GAL11 or MED2 genes abolishes this activity of the free tail domain. Interestingly, sin4Δ mutation also increases resistance to immunosuppressant drug rapamycin in a Gal11p- and Med2p- dependent manner. However, the increased resistance to rapamycin in sin4Δ cells occurs independently of Flr1p, thus underscoring the existence of multiple transcriptional targets of the free tail domain. In conclusion, our results show that the free tail domain of the Mediator is responsible for resistance to several drugs by functioning as a transcriptional co-activator that is recruited to multiple promoters.

2090/B469
Regulation of Notch1 Signaling By Runx2 during Osteoblast Differentiation.
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Notch1 genes encode receptors for a signaling pathway that regulates cell growth and differentiation in various contexts, but the role of Notch1 signaling in osteogenesis is not well defined. Notch1 controls osteoblast differentiation by affecting Runx2, but the question arises whether normal osteoblastic differentiation can occur regardless of the presence of Notch1. In our present study, we observed the down regulation of Notch1 signaling during osteoblastic differentiation. Alk6-induced Runx2 proteins reduced Notch1 activity to a marked degree. And accumulated Runx2 suppressed Notch1 transcriptional activity via the dissociation of the Notch1-IC-RBP-Jk complex. Using deletion mutants, we also determined that the N-terminal domain of Runx2 was crucial to the binding and inhibition of the N-terminus of the Notch1 intracellular domain. Notably, up-regulation of the Runx2 protein level paralleled reduced expression of Hes1, which is a downstream target of Notch1, during osteoblast differentiation. Collectively, our data suggest that Runx2 is an inhibitor of the Notch1 signaling pathway during normal osteoblast differentiation.

2091/B470
Methyl CpG Binding Protein 2- (MeCP2-) Mediated Repression of Transcription.
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Epigenetic changes, particularly hypermethylation of the promoter regions of tumor-suppressor genes, are important for cancer development. DNA methylation marks are recognized and bound by methyl-CpG-binding domain family proteins, including MeCP2. This binding leads to recruitment of regulatory proteins that translate the methylation marks into changes in histone modifications, chromatin structure and gene expression. MeCP2 expression and binding have also been linked to prostate cancer cell growth and estrogen receptor expression in neoplastic tissue. MeCP2 binds CpG-methylated DNA and represses transcription. Despite the prevalence of CpG methylation in mammalian genomes, MeCP2 selectively acts at target genes. The determinants of this selectivity have not been established. MeCP2 has been shown to co-purify with histone deacetylase- (HDAC-) containing complexes. However, MeCP2-mediated repression of transcription also acts through an as yet poorly understood HDAC-independent mechanism. In order to study both the determinants of target selection by MeCP2 and the mechanisms of transcription repression, we have established an In Vitro transcription system in which MeCP2 is active. This work is particularly exciting and novel for a number of reasons. First, the set of proteins in our HeLa nuclear extract is probably a better representation of the In Vivo environment of the nucleus than purified systems, which likely lack key cofactors found in the cell. Second, we are using wild-type MeCP2 rather than Gal4-MeCP2 fusion proteins. Third, our system is the first to achieve equivalent levels of transcription from methylated and unmethylated promoters in the absence of MeCP2. Using our transcription system, we observe potent, HDAC-independent, repression of transcription by MeCP2. We have shown that this repression occurs through inhibition of the formation of a functional transcription preinitiation complex. In addition we have
begun to elucidate the methylation patterns required for target selection by MeCP2. These studies will lead to a greater understanding of the role of MeCP2 in silencing of tumor suppressor genes and other genes important to cancer development.

2092/B471
Histone Variant H2A.Z and RNA Polymerase II Transcription Elongation.
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Nucleosomes containing histone variant H2A.Z, Htz1 in budding yeast, serve to poise quiescent genes for activation and transcriptional initiation. However, little is known about their role in transcription elongation. Although htz1Δ mutants exhibit synthetic genetic interactions with genes encoding elongation factors, and are hypersensitive to the drug 6-azauracil (6-AU), evidence for a direct role in transcription elongation is currently lacking. Here we show that dominant mutations in the elongation genes SPT5 and SPT16 suppress the 6-AU sensitivity of htz1Δ, demonstrating that this defect is at the level of transcription elongation. Measurements of the kinetics of Pol II movement across the 9.5 kb GAL10p-VPS13 gene reveal that the elongation rate of polymerase is significantly slower in the htz1Δ mutant. Finally, both the phospho-Ser2 levels in the elongating isoform of Pol II and the loading of Elongator are severely reduced over the GAL1 gene open reading frame in htz1Δ. These results establish a mechanistic role for Htz1 in transcription elongation, and suggest that Htz1-containing nucleosomes, likely within promoter proximal chromatin, serve to ensure the correct assembly and modification status of Pol II elongation complexes.

2093/B472
Recruitment of Bcl10 to H2AX Foci in Breast Cancer.
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Phosphorylated histone H2AX (γ-H2AX) foci form at sites of DNA double strand breaks (DSB) induced by ionizing radiation and at constitutive sites of DNA damage attributed to telomere dysfunction. γ-H2AX foci are highly dynamic and contain proteins involved in DNA repair and the regulation of apoptosis. Identifying proteins that associate with γ-H2AX foci can therefore provide insight into processes that determine the response of cancer cells to treatment. Using immunofluorescence confocal microscopy we observed that B cell lymphoma 10 (Bcl10), a protein linked to activation of the transcription factor nuclear factor kappa B (NF-κB) in the cytoplasm of lymphoid cells, localizes to constitutive γ-H2AX foci in the nuclei of breast cancer cells. Following exposure to 5 gray of ionizing radiation Bcl10 re-distributes from these sites to the γ-H2AX foci that form at DSB. The number of Bcl10 foci increases over time post-irradiation becoming maximal at 4 hours. Recruitment of Bcl10 therefore lags behind that of repair proteins such as ataxia telangiectasia mutated (ATM) suggesting Bcl10 does not contribute to DNA repair per-se. We observed that siRNA knockdown of Bcl10 results in loss of constitutive γ-H2AX foci, induction of apoptosis and increased radiosensitivity, indicating Bcl10 protects cancer cells from apoptosis. NF-κB is activated by ionizing irradiation and regulates transcription of genes that are anti-apoptotic. To investigate whether Bcl10 influences NF-κB activation we quantitated the effect of Bcl10 knockdown on re-distribution of the NF-κB subunit p65 from the cytoplasm to the nucleus following irradiation. We observed that Bcl10 knockdown significantly reduces re-distribution of p65 indicating Bcl10 regulates NF-κB activity in response to DNA damage. We conclude that Bcl10 may play a novel and previously unsuspected role in protecting cancer cells from apoptosis through regulation of NF-κB. By localizing to γ-H2AX foci Bcl10 could relay information regarding DNA damage to NF-κB.

2094/B473
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Retinoids, a class of compounds derived from vitamin A, can be used clinically to treat a variety of skin disorders and certain cancers. An isoform of vitamin A, 9-cis retinoic acid (9-cis RA), binds to the nuclear retinoid X receptor (RXR) and induces RXR homodimer formation and RXR-mediated transcription. Bexarotene (Bex) is a synthetic ligand (rexinoid) modeled after 9-cis RA that is indicated for the treatment of cutaneous T-cell lymphoma (CTCL). Bex can stimulate RXR homodimer formation and modulate the expression of 9-cis RA target genes. However, Bex can also dysregulate other RXR-requiring pathways since other nuclear receptors (e.g., TR and RAR) form heterodimers with RXR. Therefore, we sought to model (via docking studies) and synthesize novel analogs of Bex, including nitro- and fluoro-substituted compounds, that bind RXR and mediate regulation of anti-tumor genes, without disrupting other RXR pathways. Employing both a mammalian two-hybrid system, and an RXRE-mediated transcriptional assay, we tested 19 analogs of Bex and discovered three compounds that best induce homodimerization and RXR-mediated transcriptional activity (20-120% of Bex). These three analogs also stimulate significant apoptosis in CTCL cells, and have similar (analog 16 and 18) or better (analog 20) Ki and EC50 values when compared to the Bex parent compound. We also evaluated Bex and the three analogs for their "residual" retinoic acid receptor (RAR) agonist activity employing expression of human RAR and a retinoic acid responsive element (RARE)-luciferase reporter system and found that these three analogs are selective RXR agonists (especially analog 18). Taken together, these results suggest that modification of Bex with a halogen atom on the aromatic ring that bears the carboxylic acid may reduce the activation of RAR (analog 18), or increase its ability to activate RXR (analog 20). Based on these novel results, we have designed three new halogenated compounds, and other compounds functionalized with hydrogen bonding groups, that are being evaluated to test this hypothesis. In conclusion, our experimental approach suggests that rational drug design can be employed to develop rexinoids with improved biological properties.

2095/B474
Identification of DNA Binding Proteins Interacting with the Promoter Regions of the Bovine Oct4 and Sox2 Genes By Utilizing the Electrophoretic Mobility Shift Assay Followed By Mass Spectrometry Analysis.
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Transcription factors play a critical role in gene regulation and the characterization of these DNA binding proteins is crucial for elucidating some of the interacting proteins that assist in regulating transcription. The interaction of proteins with DNA corresponding to different portions of the promoter regions of the bovine Oct4 and Sox2 genes was evidenced by a mobility shift in the band migration compared to the control DNA. The shifted bands were excised from the 6% polyacrylamide gel and were subsequently submitted to mass spectrometry analysis for identification of candidate proteins. Putative proteins identified are involved in diverse cellular transcriptional processes including: transcriptional activation; chromatin modification, decondensation, and distribution; nuclear architecture including core histone 2a; constitutive and alternative splicing; DNA repair; regulation of RNA polymerase II; and DNA stabilization. The use of the electrophoretic mobility shift assay in conjunction with mass spectrometry analysis results in the identification of functionally relevant DNA binding proteins and sheds additional insight into the transcription factors involved in the gene regulation of the bovine Oct4 and Sox2 genes.

2096/B475
Interaction of Human Sin3B with Tieg2 Transcription Factor.
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Sin3 is a global corepressor protein known to play a versatile role in transcription, cell cycle regulation, development and differentiation. It is known to modulate the expression of diverse genes in different contexts. Well conserved from yeast to mammals, this protein has two isoforms namely, hSin3A and hSin3B. Its a modular protein that functions mainly as a scaffold protein having multiple protein-protein interaction domains and has no DNA binding activity. There are four paired amphipathic helix (PAH) containing domains in Sin3. Our study focused on investigating interacting proteins of hSinB protein. Tieg2 (transforming growth factor- 
regulated gene) protein is one such protein that was already known to interact with Sin3A protein. Tieg2 is a transcription factor that represses transcription of SMAD7 which enhances TGF-beta signaling. In addition, Tieg2 represses promoters containing SP1-like binding sites inhibiting cell growth. Using co-immunoprecipitation experiments in HEK293 and U87 cell lines, we have found that it also associates with hSin3B protein. In addition, in our study, domain analysis using yeast two hybrid assays showed that the N-terminal region of hsin3b (from amino acids 1 to 416) harbouring three PAH domains interacts very strongly with TIEG2 protein. In comparison, the hSin3B C-terminal region (from amino acids 448 to 1177) had weak interaction with Tieg2 protein. The interaction of the corepressor hsin3b with Tieg2 transcription factor is hypothesized to have important implications in control of cell growth.

2097/B476
Nuclear Receptor and Coactivator Interaction Probed By Fluorescence Fluctuation Spectroscopy Inside Living Cells.
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The actions of nuclear receptors (NR) on gene transcription require a highly coordinated interaction with coactivators. It is generally accepted that each coactivator molecule recruits two NRs to initiate gene activation. A direct experimental investigation of the interaction between NRs and coactivators in cells is still lacking. In this work, we apply dual-color time-integrated fluorescence cumulant analysis (TIFCA), a fluorescence fluctuation spectroscopy technique, to investigate the interaction between the coactivator TIF-2 and two nuclear receptors, retinoid acid receptor (RAR) and retinoid X receptor (RXR), directly in living cells. We measure full length RAR and RXR and find that TIF-2 indeed recruits dimers of full length NRs. Since it is known that RXR forms heterodimer with RAR, we will further test whether heterodimer RXR/RAR has advantages over RXR homodimer when recruiting into the coactivator complexes. This study demonstrates that TIFCA reveals the composition of heterocomplexes involving three proteins directly inside living cells. This work is supported by the National Institutes of Health (R01 GM64589).

Oncogenes and Tumor Suppressors (2098 – 2112)

2098/B477
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The transcription factor p63 belongs to the p53 protein family and plays an important role in epithelial development. However, recent studies showed that p63 is over-expressed in some human squamous cell carcinomas of the head and neck, suggesting a role in carcinogenesis. In this study, we show that the expression of TAp63 gene is upregulated in p53 deficient human cancer cell lines after treatment with doxorubicin, a widely used chemotherapeutic drug. In contrast, treatment of doxorubicin has no effect on TAp63 promoter activity in p53-positive cell lines. We further show that the growth suppressive effect of doxorubicin is mediated, at least in part, through upregulation of TAp63, which results in an increase of the G1 phase cell. In addition, knockdown of TAp63 expression leads to increased Hep3B cell proliferation and decreased inhibition by doxorubicin. Reporter assay show that the upregulation of TAp63 promoter activity is correlated with increased c-jun expression and decreased c-jun
phosphorylation. Moreover, enhanced TAp63 expression is coincided with an increased binding of c-jun to the TAp63 promoter. Point mutation of the sp1 binding site within the TAp63 promoter region attenuated the effect of c-jun on TAp63 induction. Taken together, the growth suppression effect of doxorubicin in p53 negative cancer cells appears to be mediated, at least in part, through the enhanced expression of TAp63. Thus, TAp63 may function as a tumor suppressor in p53 negative tumors.

2099/B478
Human Homolog of Drosophila Hairy and Enhancer of Split 1, Hes1, Negatively Regulates δ-Catenin (CTNND2) Expression in Cooperation with E2F1 in Prostate Cancer;
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Neuronal synaptic junction protein δ-catenin (CTNND2) is often overexpressed in prostatic adenocarcinomas but its mechanisms of activation are quite variable. Here we report that Hes1, human homolog of Drosophila Hairy and enhancer of split (Hes) 1, is a transcriptional repressor of δ-catenin expression. In δ-catenin promoter reporter assay, Hes1, but not its inactive mutant, inhibited the upregulation of δ-catenin-luciferase activities induced by E2F1. While δ-catenin overexpression in prostate cancer cell lines cannot be attributed to the deregulation of any single transcriptional activators or repressors, the ectopic overexpression of Hes1 reduced the expression of δ-catenin. The interruption of Notch signaling using γ-secretase inhibitors to reduce Hes1 expression increased δ-catenin expression, altered cell cycle progression, and promoted cell shape changes in prostate cancer cells. In neuroendocrine prostate cancer mouse model derived allograft NE-10 tumors that showed increased δ-catenin expression, E2F1 transcription was high when Hes1 displayed residual expression whereas it was low when Hes1 expression was completely suppressed. These studies demonstrate coordinated regulation of δ-catenin expression by both activating and repressive transcription factors in prostate cancer progression.

2100/B479
The Growth Suppressor and Ras-Binding Protein NORE1A Regulates Microtubule Nucleation.
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NORE1A (Novel Ras Effector 1 isoform A, also known as RASSF5) is a protein capable of binding activated Ras and Ras-related proteins In Vitro and in vivo. NORE1A is expressed in most normal tissues but is lost in many cancers. Reconstitution of NORE1A expression induces growth arrest as well as cell death in a variety of cell lines. However, the molecular mechanism by which NORE1A induces growth suppression is unknown. We have shown previously that NORE1A might be involved in regulation of microtubule dynamics. The closest NORE1A relative, RASSF1A growth and tumor suppressor, was shown to be involved in regulation of stability of interphase microtubules. To examine if NORE1A has a role in microtubule stabilization, we expressed GFP-NORE1A in A549 cells and induced microtubule depolymerization by cold treatment. The GFP-RASSF1A served as a control. We found that, contrary to RASSF1A expression, NORE1A expression did not result in microtubule stabilization. To determine if NORE1A is involved in regulation of microtubule nucleation in interphase cells, we depleted NORE1A by RNA interference, depolymerized microtubules with nocodazole and examined microtubule nucleation after nocodazole washout. We found that, in primary human WI-38 fibroblasts, NORE1A depletion markedly reduces microtubule nucleation by non-centrosomal sources, specifically by the Golgi vesicles. Since NORE1A binds activated Ras, we examined the effect of Ras on microtubule nucleation. The expression of activated Ras in WI-38 fibroblasts nearly eliminated both centrosomal and non-centrosomal microtubule nucleation after nocodazole
washout. Interestingly, if NORE1A was depleted in cells expressing Harvey-Ras (H-Ras), the microtubule nucleation after nocodazole washout was partially restored. This suggests H-Ras-induced suppression of microtubule nucleation is in part mediated by NORE1A. Our data suggest NORE1A directly couples Ras signaling with microtubule cytoskeleton. We propose that at certain conditions NORE1A is capable of association with Ras proteins and inhibition of microtubule nucleation. This inhibition might lead to the suppression of pre-cancerous cell growth or induction of apoptosis.

2101/B480
Cyclin G2 Is Upregulated By Estrogen Signal Deprivation and Interacts with the Endocrine Therapy-Sensitivity Biomarker, CDK10.
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Cellular proliferation in breast cancers (BCs) is driven by mitogenic signaling downstream of activated estrogen (E2) receptor (ER) in 65% of BCs. Despite clinical success of E2 and ER targeting therapeutics, resistance to current therapeutics eventually develops in a majority of BC patients, limiting the long term options for successful abatement of breast cancer metastasis. Cyclin G2 (CycG2), an unconventional negative regulator of cell cycle progression is repressed by the active E2/ER and HER2 signaling pathways in BC cells (BCCs) and thus may be a useful biomarker. We determined that CycG2 is a centrosome associated nucleocytoplasmic shuttling protein that associates with protein phosphatase 2A (PP2A) and induces a G1/S-phase cell cycle arrest when ectopically expressed. Even modest elevation of CycG2 inhibits BCC proliferation. Our recent evidence indicates that anti-estrogen SERM and SERD therapeutics enhance CycG2 expression in responsive, ER positive BCCs. Although CycG2 possesses a N-terminal ‘Cyclin Box’ domain required for activation of CDKs by prototypical cyclins, no active CycG2-CDK complex has been identified, whereas CycG2 does associate with active PP2A via its C-terminal domain. Recent reports of others showed that loss of an unconventional “orphan” CDK, CDK10, is linked with BC cell resistance to E2-deprivation and tamoxifen treatment. Significantly, we found that CycG2 robustly interacts with CDK10. Mapping analysis determined that this interaction requires the CycG2 cyclin box domain. Our studies indicate that CycG2 accumulates in the nucleus when coexpressed with CDK10, a known nuclear protein. Moreover, our experimental results support the idea that endogenous CDK10 associates with endogenous CycG2 in BC cell lines. Interestingly our additional mapping analysis suggests that CycG2 can form ternary complexes with CDK10 and PP2A. Importantly our preliminary data suggests that CycG2-CDK10 complexes contain kinase activity. We are investigating the significance of these observations and whether CycG2 modulates BC cell sensitivity to E2/ER and HER2 targeting therapeutics.

2102/B481
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In human Papillomavirus positive cervical cancer, E6/E6AP complex is mainly responsible for the degradation of tumor suppressor p53. E6/E6AP-mediated p53 degradation is well-known to be the critical mechanism for cervical carcinogenesis associated with high risk HPV. However, the regulation of p53 toward E6/E6AP-mediated degradation is still poorly studied. We report herein that high mobility group B2 (HMGB2), a non-histone nuclear factor, can inhibit E6/E6AP-mediated ubiquitination and subsequent degradation of p53 thereby increasing the stability of p53 protein in HPV positive HeLa cells. Overexpression of HMGB2 induced the accumulation of the p53 protein, whereas HMGB2-knockdown by siRNA substantially decreased the level of p53 protein in HeLa. Ectopic expression of HMGB2 also reduced the turnover of p53, which was examined by cyclohexamide chase analysis. The HMGB2-dependent increase of p53 stability was specific for
HPV-positive HeLa cells because HMGB2 did not elicit the stabilization of p53 in HPV-negative cells such as HCT116 and MCF7. Co-expression of HMGB2 and E6 prevented p53 from E6-mediated degradation in Hela and p53-null H1299 cells. Furthermore, altered expression of HMGB2 by gene silencing or overexpression demonstrated that HMGB2 blocks E6-mediated ubiquitination of p53. FACS analysis revealed that HMGB2 transfection in HeLa cells caused the decrease of cell proliferation with the concomitant increase of p53 and the cell cycle of these cells was arrested predominantly in G1 phase. Taken together, our findings suggest that HMGB2 stabilizes p53 by interfering with E6/E6AP-mediated p53 degradation in HPV-positive HeLa cells.

2103/B482
NLBP, a Novel LZAP-Binding Protein, Functions as an Inhibitor of Cell Invasion.
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LZAP was recently shown to function as a tumor suppressor through inhibition of the NF-kB signaling pathway. LZAP is also known as a negative regulator of cell invasion, and its expression was demonstrated to be reduced in several tumor tissues. However, the molecular mechanism of the negative effect of LZAP on cell invasion is unclear. In this study, we identified NLBP as a novel LZAP-binding protein using tandem affinity purification. We demonstrate the negative effects of NLBP on cell invasion and the NF-kB signaling pathway. NLBP expression was not detected in hepatocellular carcinoma which has strong invasive activity, while its expression was detected in hepatocellular carcinoma with no invasive activity. In addition, NLBP expression was reduced in several tumor tissues. We also demonstrate that these two proteins affect each other’s stabilization by inhibiting ubiquitination of the other protein. Based on these results, we suggest that NLBP may act as a novel tumor suppressor by inhibiting cell invasion, blocking NF-kB signaling and by increasing the stability of the LZAP protein.

2104/B483
Breast Cancer Amplified Sequence 2, a Novel Negative Regulator of the P53 Tumour Suppressor.
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Breast cancer amplified sequence 2 (BCAS2) was previously reported as a transcriptional coactivator of the estrogen receptor. Here, we report that BCAS2 directly interacts with p53 to reduce p53 transcriptional activity by mildly but consistently decreasing p53 protein in normal condition. However, in the presence of DNA damage, BCAS2 prominently reduces p53 protein and provides protection against chemotherapeutic agent such as doxorubicin. When deprivation of BCAS2 in p53 wild type (wt) cells induces cell apoptosis; but in p53-null or p53 mutant cells causes G2/M arrest. There are at least two reasons to illustrate the apoptosis mechanisms when silencing BCAS2 in wt p53 containing cells: firstly, increases p53 retention in the nucleus triggered the expression of apoptosis-related genes; and secondly, affects p53 phosphorylation at Ser 46 responsible for p53-targeted gene expression and Ser 315 for p53 protein degradation. We demonstrate for the first time that BCAS2, a small nuclear protein (26 kDa), is a novel negative regulator of p53, and hence a potential molecular target for cancer therapy.

2105/B484
Novel Binding Partners of Cdk6.
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Cyclin dependent kinase (cdk)6, is known to regulate G1 phase of the cell cycle by partnering with D-type cyclins to phosphorylate pRb. Historically, the role of cdk6 has been understood to be
redundant with that of cdk4, a homologous D-type cyclin. However, recent evidence from several labs using a variety of model systems has shown an emerging role for cdk6 in the process of differentiation. Importantly, this function is not shared with cdk4, exposing for the first time meaningful functional differences between these D-cyclin kinases. There have been reports of cdk6 binding transcription factors that are important in differentiation, and it is thought that this may be one way that cdk6 acts to block differentiation. Work in our lab has demonstrated that cdk6 binds to a novel developmentally important transcription factor that we identified in a yeast two-hybrid screen. Recent GST-binding studies have confirmed the initial binding identified in the yeast screen. Preliminary data demonstrates competition for binding with a co-factor of the developmentally important transcription factor. Work is currently underway to determine if this transcription factor binds to other cyclin dependent kinases and/or to cdk6 mutants. To identify mutants of cdk6 that disallow binding of cdk6 and the transcription factor, a cdk6 mutant library is being screened. This library is being screened using the two-hybrid system to test for loss of binding. This work that is aimed at discerning mechanism of cdk6 function in the process of differentiation.

2106/B485
ANXA7: A Negative Regulator of the EGFR Signaling Pathway.
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Background: Glioblastoma is a devastating brain tumor and a complex disease. Our previous studies have shown that the co-selection of amplification of the EGFR oncogene and monosomy at chromosome 10q21.1-q21.2 might be due to haploinsufficiency of ANXA7, a tumor suppressor & positive regulator of EGFR signaling. Method: We assesses the physiological function and effect on EGFR signaling and tumorigenicity of two ANXA7 (VA/VB) splicing variants (VB: full-length variant, VA: spliced variant lacking cassette exon 6) in glioblastoma via pyrosequencing and cloning & over expression of both ANXA7 variants into glioblastoma cell lines with ANXA7 wild-type and deletion status. Result: Compared to normal cells, neural stem cells, and brain tumor stem cells, a subset of glioblastomas demonstrates increases expression of ANXA7VA compared to ANXA7VB. We found that both ANXA7 variants complex with EGFR, but demonstrate a distinct pattern of protein stability. Only overexpression of ANXA7VB in glioblastoma cells with heterozygous ANXA7 deletion, but not of the less stable ANXA7VA variant, reduces EGFR protein abundance and attenuates EGFR down stream signaling (PI3Kinase/Akt, MAPKinase pathways). ANXA7VB over expression enhances EGFR ubiquitination and is associated with decreased tumorigenic potential. In turn, knockdown of ANXA7VB in glioblastoma cells augments activating EGFR phosphorylation. Similar results were obtained with human embryonic 293T kidney cells suggesting a physiological role of ANXA7 in EGFR regulation. Conclusion: Anxa7 is a bona fide tumor suppressor that terminates EGFR signaling through EGFR ubiquitination.

2107/B486
YAP Is the Effector of the Hippo Signaling Pathway and Uses Its WW Domains and PDZ Binding to Regulate Apoptosis.
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The HIPPO pathway regulates the size of organs by controlling two opposing processes: apoptosis and proliferation. YAP, Yes kinase-associated protein, is a WW domain-containing transcriptional co-activator that acts as the effector of the HIPPO pathway in mammalian cells. In addition to WW domains, YAP has a PDZ-binding motif at its C- (Carboxy)-terminus. Both the WW domains and the C-terminal PDZ-binding motif are well conserved and regulate YAP through cognate complexes. The two WW domains of YAP act in concert to enhance the apoptotic function of YAP by forming a functional complex with a pro-apoptotic member of the p53 family, p73, when cells are affected by stress conditions such as 1% serum or DNA damaging agents. The YAP-p73 complex results in the stabilization of p73. The C-terminal sequence of YAP is required for the nuclear translocation. This process is mediated by a tight junction protein, ZO-2, which binds to the C-terminus of YAP via its first PDZ domain and serves as the YAP shuttle. The endogenous ZO-2 and YAP proteins co-localize in the nucleus. The nuclear localization and pro-apoptotic function of YAP is PDZ-domain dependent. This is the first report on the PDZ-based nuclear translocation mechanism. Since the HIPPO pathway is a tumor suppressor pathway, the YAP-ZO-2 complex represents a target of cancer therapy. Small molecules blocking YAP WW and PDZ domain complexes could be tailored to inhibit proliferation of cancer cells, and redirect them toward apoptotic death.

2108/B487
Cell Cycle-Related Kinase Phosphorylates Casein Kinase Beta in Glioblastoma Cells.
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Our laboratory has demonstrated that cell cycle-related kinase p42 (CCRK) is involved in glioblastoma carcinogenesis. Overexpressing CCRK confers tumorigenicity to a non-tumorigenic U-138 MG cell line, and the expression of CCRK is up-regulated in glioblastoma patient samples and a number of glioblastoma cell lines. Suppression of CCRK by siCCRK inhibited the proliferation of U-373 MG and U-87 MG glioblastoma cells in a time- and dose-dependent manner[Ng et al., 2007]. In addition, siCCRK transfection sensitized glioblastoma cells to chemotherapeutic drugs like cisplatin. However, the mechanism under its oncogenicity and its mediated drug resistance, as well as its interacting partner or substrate in glioblastoma is unknown. In order to resolve this problem, we conducted yeast-two hybrid (Y2H) recently and identified that casein kinase 2 is one of the interacting proteins of CCRK. By cell fractionation and western blotting, we found that casein kinase 2 beta phospho S209 was increased significantly in nucleus but not in cytoplasm of glioblastoma U373 and U87 cells after overexpressing CCRK. Therefore we hypothesize that cell cycle-related kinase phosphorylates casein kinase 2 and promotes casein kinase shuttling to nucleus, may in turn confers oncogenicity and drug resistance to glioblastoma cells. Reference: Ng S, Cheung Y, An X, Chen Y, Li M, Hoi-Yee Li G, Cheung W, Sze J, Lai L, Peng Y. 2007. Cell cycle-related kinase: A novel candidate oncogene in human glioblastoma. JNCI Journal of the National Cancer Institute 99:936-948.

2109/B488
DN-Mutant p53 Induces Cell Proliferation Linked to Loss of Epithelial Cytokeratin 18 and Actin Reorganization in Human Breast Carcinoma Cells.
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INTRODUCTION. - Progression of carcinomas involves loss of epithelial cytoarchitecture from a cobblestone appearance to a mesenchymal phenotype, producing an epithelial to mesenchymal transdifferentiation (EMT) (1). In normal mammary epithelium, luminal cells usually express cytokeratin 18 (CK 18) However, CK18 is down-regulated with progression and metastasis of breast cancers (2). Concomitant loss of cytoskeletal CK18 (2) and E-cadherin (3) may favour actin reorganization, facilitating proliferation (1-3). OBJECTIVES.- Since the p53 tumor suppressor pathway is inactivated in most human cancers due to gene mutations or defective signalling, we investigated in human wt p53 breast carcinoma MCF-7 cells, whether CK18 and actin are modified by a dominant-negative (DN) R175H mutant p53 (3). This mutation occurring in the p53 zinc-binding domain, is common to breast cancer patients poorly responsive to
therapy. METHODS. - Fluorescence microscopy was used to determined changes in cytokeratin 18, further confirmed by immune blotting. Laser scanning cytometry was used to determine actin reorganization and cell proliferation. RESULTS. - Cells retrovirally transduced with the DN-R175H mutant p53, revealed extensive loss of CK18, increased actin organization and greater proliferation. CONCLUSIONS. - These results suggest that actin reorganization and loss of CK18 associated with the aggressive behavior of breast cancer cells (2) are mediated by the gain of oncogenic function induced by DN-R175H mutant p53 in carcinomas (3) REFERENCES 1.-Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009 119:1420-8. 2.- Woelfle U et al. Down-regulated expression of cytokeratin 18 promotes progression of human breast cancer, Clin. Cancer Res. 2004, 10:2670-4. 3.-Rieber, M. and Strasberg-Rieber, M. DN - R175H p53 mutation is more effective than p53 interference in inducing epithelial disorganization in human carcinoma cells: Role of E-cadherin. Int J Cancer 2009, 125:1604-12. * Research supported by FONACIT -Mision Ciencia, Subproject SPNS N°4, Cancer

2110/B489


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Cell cycle control plays an important role in maintaining the balance of T-lymphocyte (T cell) proliferation, anergy and apoptosis, which is central to immune function. The tumor suppressor gene Zac1 encodes a novel seven-zinc finger protein which shares with p53 the ability to regulate apoptosis and cell cycle arrest concurrently. Imprinted expression of Zac1 has been demonstrated in many human and mouse tissues, although biallelic transcription has been noted in human peripheral blood leucocytes. Zac1 (=Plagl1) has recently identified as a signature of regulatory T cells - a subtype of T cells (Hill JA et al. Immunity. 2007). Bioinformatics analysis using the immunological genome (http://www.immgen.org/) revealed that the leucocytes expression pattern of Zac1 was very similar with another important cell cycle regulator, cytotoxic lymphocyte-associated antigen-4, which is associated with autoimmune diseases (Ueda H. et al. Nature 2003). Toward a better understanding to regulate T cells, Zac1 protein induction was screened with a variety of signal inhibitors, stimulators, anti-microtubules drugs and immunosuppressive drugs etc. by the western blot analysis in T cell-lines. Zac1 protein was induced by a calcineurin-inhibiting immunosuppressive drug cyclosporin a in T cell-lines. Zac1 protein was also induced by the transforming growth factor-β treatment. Real-time PCR is being performed by using Taqman probes and the Applied Biosystems StepOnePlus™. Question, is being pursued, how Zac1 is precisely regulated in T cells in the relationship with other important T cell regulators such as p21, p27 and the forkhead-winged helix transcription factor Foxp3.

2111/B490

The FOXO Regulated Cell Cycle Inhibitor, Cyclin G2, Is Upregulated by mTOR Inhibition and Opposed by Ubiquitin-Mediated Degradation.

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Cyclin G2 (CycG2) is an unconventional cyclin linked to cell cycle arrest encoded by the CCNG2 gene. Several pro-growth signaling pathways and transcription factor (TF) complexes suppress CCNG2 expression, whereas the PI3K/AKT-targeted TFs FOXO1 and FOXO3 directly activate CCNG2 expression upon inhibition of PI3K/AKT signaling. We previously showed that CycG2 forms enzymatically active complexes with protein phosphatase 2A and that ectopic expression of CycG2 induces a p53-dependent G1/S-phase cell cycle arrest. Through collaborative studies we determined that CycG2 is repressed by HER2-driven PI3K/AKT/mTOR signaling in breast cancer (BC) cells, and mTOR activity in embryonic stem cells. We and others found that expression of CycG2 is also modulated at the post-translational level through ubiquitin-mediated proteasomal degradation. Skp2, the targeting subunit of the SCF E3 ubiquitin-ligase complex, was recently reported to associate with the C-terminus of CycG2. Importantly Skp2 is an oncogenic protein
found over-expressed in BC and B-cell lymphoma cells that also targets FOXO TFs for degradation. We hypothesize that reduced expression of CycG2 promotes cellular proliferation and enhances growth of BC and B-lymphocyte derived tumors. Here we investigate CycG2 expression and localization during growth inhibitory signaling in BC and B-cell lymphoma cell lines. Suppression of mTOR signaling via rapamycin lead to increased CycG2 protein expression coincident with inhibition of cell cycle progression of tumor cells. The sensitivity of diffuse large B-cell lymphoma lines to rapamycin correlated with cyclin G2 abundance. Using serially truncated CycG2 constructs co-expressed with a tagged form of ubiquitin we show that the region in CycG2 most sensitive to ubiquitination lays within the 45 AA residues immediately C-terminal to its 'Cyclin-Box' domain. Our results suggest that two C-terminal regions in CycG2 mediate the interaction with Skp2 and that this association alters the localization of CycG2. Through RNAi and construction of ubiquitination-resistant mutants of CycG2 we seek to determine the influence of CycG2 stability on tumor cell growth and responsiveness to therapeutics.

2112/B491
MicroRNAs Regulating Matrix Metalloproteinases-2 Activities.
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Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, remodel and degrade the extracellular matrix (ECM). Twenty eight MMPs have been identified to date and MMPs are classified based on their substrate specificity and domain organization. for example, MMP-2 and MMP-9 are classified into gelatinase because they can degrade gelatin. Tumor cell invasion is prerequisite to metastasis and MMPs, especially MMP-2 and MMP-9, play an important role in tumor cell invasion. However, molecular mechanism underlying tumor cell invasion remains elusive. Recently, miRNAs were reported to be implicated in tumor cell invasion and metastasis. To identify miRNAs regulating MMP-2 activities, we screened 215 miRNAs for upregulating MMP-2 activities in human fibrosarcoma HT1080 cells using gelatin zymography. Several miRNAs increased MMP-2 activities and tumor cell invasion, and the increase was further heightened by double or triple transfection of those miRNAs. Candidate target genes of those miRNAs were identified by bioinformatics and some of them were verified by luciferase assay.

Apoptosis (2113 – 2131)

2113/B492
Survival Effect of Hypoxia Induced Gene-1a (HIG-1A) Is Associated with the Reduced Activities of Caspases 9.
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Hig-1a is a novel gene that can be induced in the condition of hypoxia, deprivation of glucose, and serum starvation. It is a 10.23kDa and mitochondrial inner membrane protein, which is oriented with N’ outside-C’ outside and loop inside. There are 4 different types of Hig found in Human, Hig-1a, Hig-1b, Hig-1c and Hig-2a. Recently, over-expression of human Hig-1a cells was reported to induce survival effect against stimuli of hypoxia and low glucose in pancreatic cells(PNAS 103, 10636-10641). In present study, we provide evidence that the survival effect of Hig-1a is related with caspase 9 activities. First, we cloned 500 bp upstream region of Hig-1a that has a strong transcriptional activity for Hig-1a to test transcriptional regulation. It has HRE (hypoxia response element) sites. Among them, HRE (-168～164) is the responsible element for the transcriptional activity. Mutation of the site could abolish the transcription of Hig-1a induced by hypoxia and CoCl2. AsPc-1 cells transfected with Hig-1a underwent less cell death than control
cells in hypoxic condition. Whereas, cell growth was severely retarded in normoxia when endogenous Hig-1a was reduced by transfection with siRNA, Lastly, the survival effect induced by Hig-1a is related with reduced activities of Caspase 9. Collectively, our data demonstrate that transcription of Hig-1a is induced in a HIF-1-dependent fashion, and that the survival effect of Hig-1a against hypoxia is mediated by preventing activation of caspases 9.

2114/B493
Nestin Is a Critical Prosurvival Factor in Neural Stem/Progenitor Cells.
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The intermediate filament protein, nestin, is a widely employed marker of multipotent neural stem/progenitor cells (NSCs). Recent In Vitro studies have implicated nestin in a number of cellular processes, but there is no data yet on its In Vivo function. Here, we report the construction and functional characterization of Nestin knockout mice. We found that these mice show embryonic lethality, with neuroepithelia of the developing neural tube exhibiting significantly increased apoptosis. Consistent with this In Vivo observation, NSC cultures derived from knockout embryos show dramatically elevated apoptosis but no defects in proliferation or differentiation. Unexpectedly, nestin deficiency has no detectable effect on the integrity of the cytoskeleton. Furthermore, the knockout of Vimentin, which abolishes nestin’s ability to polymerize into intermediate filaments in NSCs, does not lead to any apoptotic phenotype. These data demonstrate that nestin is a critical prosurvival factor in NSCs, and that nestin’s prosurvival function is surprisingly uncoupled from its structural involvement in the cytoskeleton.

2115/B494
Cytolytic Peptides Carried By Molecularly Targeted Nanoparticles Induce Apoptosis in Human Endothelial Cells.
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Melittin is a 26 amino acid amphipathic, pore-forming peptide that comprises the majority of the venom from European honeybees (Apis mellifera). The lytic properties of melittin have been recognized for quite some time, and when administered intravenously in free form can cause profound hemolysis. By incorporating melittin into a perfluorocarbon nanoparticle, off-target effects can be alleviated and the peptide safely delivered to specific sites of interest. Nanoparticles on the order of 200nm in size generally remain in the vascular system such that if they were to be used for cancer therapeutics, their ability to either deliver drug beyond the endothelium or at least kill the tumor neovasculature would be useful to document. Previous work in our laboratory has demonstrated that nanoparticle-delivered melittin successfully kills syngeneic mouse and human tumor cell implants, and at the same time attenuates angiogenesis. To define one possible mechanism by which nanoparticle-delivered melittin could eliminate tumors by targeting endothelial cells, low passage human umbilical vein endothelial cells (HUVECs) were treated with perfluorocarbon nanoparticles containing a targeting ligand directed to αvβ3 integrin with the equivalent dose of 12.5μM of melittin. A nanoparticle binding assay was done to demonstrate selective targeting of the αvβ3 particles. To assess for apoptosis and necrosis, AnnexinV/Topro-3 staining and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed after melittin treatment. The results of the binding assay suggest that HUVECs manifest sufficient expression of αvβ3 to enable nanoparticle targeting. The TUNEL assay confirmed that melittin induces apoptosis when delivered by a targeted nanoparticle. The AnnexinV/Topro-3 staining results demonstrate that the mechanism of cell death is distinguishable from necrosis which is observed when free melittin is incubated at equal
concentrations in culture media. Taken together, these results suggest that nanoparticle-delivered melittin when targeted specifically to αvβ3 integrins, which are often upregulated in tumor angiogenesis, can induce apoptosis in human vascular endothelial cells.

2116/B495
Humanin - a Rescue Peptide for Oxidative Stress: Implications for Atherosclerosis.
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Background and objective: Humanin (HN) is a recently identified peptide isolated from brain tissue of patients with Alzheimer disease (AD) and found to suppress neuronal cell death induced by various insults related to AD. Atherosclerosis, similar to AD, is an age related disorder where oxidative stress plays a major role in altering cell function. The early stage of atherosclerosis is characterized by endothelial cell dysfunction, penetration of LDL into the vascular wall, and oxidation of LDL, leading to oxidative stress and inflammation. Here we explored the expression of HN in the vascular wall, and its ability to protect human aortic endothelial cells (HAECs) from OxLDL. Results: Our results show that HN is expressed in the human vasculature bed at the endothelial cell layer. In order to test the hypothesis that HN has a protective effect in endothelial cells against oxidative stress, we incubated cultured HAEC with HN and triggered the process of reactive oxygen species (ROS) generation and apoptosis by exposure to OxLDL. Cells incubated with HN prior to OxLDL treatment displayed a significant decrease in ROS production (50%) in a dose-dependent manner. Extending the exposure time of cells to OxLDL to 6 hours initiated apoptosis, however in cells pre-incubated with HN, the number of apoptotic cells was reduced by 50%. We further investigated the mechanism underlying the role of HN in the defensive anti-oxidative mechanism of the vasculature system. Since one of the main biological effects of OxLDL is the increase of cellular levels of ceramide, a sphingolipid that mediates apoptosis, we monitored by mass spectrometry the change in different species of ceramide in cells untreated or pretreated with HN followed by exposure to OxLDL. HN pretreatment resulted in decreased levels of several species of long ceramides (e.g. C24) that are known to be involved in apoptosis, suggesting an anti-apoptotic mechanism in which HN inhibits the usual generation of ceramide due to oxidative stress. Conclusion: This study is the first to demonstrate that HN is present at the vascular wall in humans and may have an important protective role in atherosclerosis by attenuating oxidative stress by reducing ROS production.

2117/B496
8-Cl-cAMP Induced Growth Inhibiton and Apoptosis in LS 174T Cells Is Mediated through ERK Mitogen-activated Protein Kinase.
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8-Cl-cAMP (8-chloro-cyclic AMP), which induces differentiation, growth inhibition and apoptosis in various cancer cells, has been investigated as a putative anti-cancer drug. However, the action mechanism of 8-Cl-cAMP is still uncertain. Previously, we reported that 8-Cl-cAMP induce growth inhibition and apoptosis through p38 mitogen-activated protein kinase (MAPK) activation in many cancer cell lines. However, in this study, we found a requirement for ERK, a member of the mitogen-activated protein kinase family in mediating 8-Cl-cAMP-induced growth inhibition and apoptosis of human colorectal adenocarcinoma LS 174T cells. To investigate that ERK plays an important role during the 8-Cl-cAMP-induced growth inhibition and apoptosis in LS 174T, we used three MAPK inhibitors in combination with 8-Cl-cAMP. FR180204 (a ERK-specific inhibitor) recovered the 8-Cl-cAMP-induced growth inhibition and apoptosis, whereas other MAPK inhibitors, such as SB203580 (a p38-specific inhibitor) and SP600125 (a c-Jun NH2-terminal kinase-specific inhibitor), had no effect in LS 174T cells. The phosphorylation (activation) of ERK
MAPK was increased in a time and dose-dependent manner after 8-Cl-cAMP treatment in LS 174T cells. Furthermore, we showed that 8-Cl-cAMP increased ERK 1/2 phosphorylation, which was inhibited by ABT702 (Adenosine kinase inhibitor) and NBTI (Adenosine transporter inhibitor) in LS 174T cells. Also, FR180204 was able to block the 8-Cl-cAMP-induced apoptosis, which was assessed by PARP (poly-(ADP-ribose) polymerase) cleavage in LS 174T cells. These results suggest that ERK MAPK activation plays an important role in mediating 8-Cl-cAMP-induced growth inhibition and apoptosis in LS174T cells.

2118/B497
PA28γ Mediated Degradation of p38 MAPK Reduces Survival Following Cellular Stress.
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PA28γ is a nuclear proteasome activator that facilitates protein degradation by the 20S proteasome in an ATP- and ubiquitin-independent manner. Some targets of PA28γ are key cell cycle regulators such as p21Cip1, p16INK4a, p19ARF, and p53, however PA28γ also has an essential role in controlling apoptosis demonstrating its unique potential in the development of cancer treatments. This in turn has inspired exploration of mitogen-activated protein kinase (MAPK) pathways, which have a well characterized role as regulators of cell growth, differentiation, and apoptosis. The role of the p38 MAPK pathway in particular, has proven to be controversial in apoptosis, varying according to stimuli and cell type. Our objective in this study has been to further investigate PA28γ and its role in the regulation of apoptosis through its relationship with the stress-survival p38 MAPK pathway. Initially, we identified increased expression of p38 MAPK in PA28γ−/− murine embryonic fibroblasts (MEFs). We also revealed that p38 has a longer half life under normal and UV-stressed conditions in PA28γ+/− MEFs. Addition of the potent p38 inhibitor, SB202190, in conjunction with UVB irradiation demonstrated that p38 is important in cell survival. Furthermore, PA28γ+/+ cells demonstrated increased susceptibility to SB202190. These results suggest that PA28γ plays a role in the degradation of the p38 MAPK, thus, inhibiting a stress response survival pathway and promoting apoptosis.

2119/B498
Identification of Cell Death Regulators in Drosophila.
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Cell death is an important mechanism essential for the survival and development of many organisms. In humans, cell death is vital for the killing of nonfunctional or defective cells that otherwise may proliferate and contribute to the formation of cancers. However, too much cell death in humans could lead to degenerative disorders such as Parkinson’s disease. The great advances in Drosophila genetics allow the study of regulators of cell death in the Drosophila ovary where programmed cell death occurs at different stages of oogenesis. During late oogenesis, developmental programmed cell death occurs as the oocyte forms. Cells that nurse the oocyte condense, dump their cytoplasmic contents in the oocyte, and eventually go through cell death. The regulating pathways of this type of cell death are unknown. An unbiased misexpression screen based on EY P-elements was conducted to identify genes that regulate or play a role in cell death during late oogenesis. Two genes were found to be involved in cell death, showing an abnormal phenotype where the nurse cells fail to die or the cytoplasmic content does not get “dumped”. Very little is known about these genes thus, further characterization is being done. Through the use of molecular markers, organelles involved in cell death will be labeled and observed. The presence of these different organelles in the abnormal egg chambers will reveal what processes are taking place. This could lead to understanding the functions of the misexpressed genes and their role in inducing cell death.

2120/B499
Failure to Degrade Poly(ADP-Ribose) Polymer Causes Chromatin Structural Change, Increased DNA Damage, and Increased Apoptotic Signaling.
The coordinated synthesis and degradation of poly(ADP-ribose) (PAR) polymer is essential for genomic stability. Previously, we reported that the absence of PAR hydrolysis leads to elevated levels of PAR-modified proteins and causes hypersensitivity to DNA damage. OBJECTIVE: to elucidate the role of PARG in normal nuclear function and in response to genotoxic stress. METHODS: We utilized PARG null trophoblast stem (TS) cells, which are only viable when cultured with the PAR polymerase (PARP) inhibitor benzamide (BZ), in our studies. Chromatin structure was analyzed by treatment of nuclei with micrococcal nuclease and the DNA intercalating agent acridine orange. DNA damage was assessed by single-cell gel electrophoresis (Comet) assay following sublethal doses of the DNA alkylating agent MNNG. Because PAR can translocate from the nucleus to the cytoplasm, where it signals the mitochondrial release of apoptosis-inducing factor (AIF), cytoplasmic PAR levels were analyzed by subcellular fractionations and immunodetection. RESULTS: Increased DNA laddering was observed in PARG null TS cells -BZ, indicating greater susceptibility to micrococcal nuclease digestion than wild-type. Further, FACS analysis demonstrated a greater amount of acridine orange DNA intercalation in PARG null TS cells -BZ. These results suggest greater access of DNA-modifying agents to genomic DNA in PARG null TS cells. This was further demonstrated by treatment of TS cells with a minimal dose (5 µM) of MNNG, which resulted in massive amounts of DNA damage in PARG null TS cells -BZ. Finally, high levels of cytoplasmic PAR were detected in PARG null TS cells -BZ, indicating that apoptotic signaling was up-regulated following the failure to degrade PAR. CONCLUSIONS: The results demonstrate that elevated levels of nuclear PAR due to the absence of PARG lead to chromatin decondensation, which allows greater access of DNA-damaging agents to genomic DNA. This greater access leads to increased DNA damage, while elevated nuclear PAR levels lead to increased cytoplasmic PAR cell death signaling. Taken together, the results suggest essential roles for PARG in chromatin superstructure, the response to DNA damage, and the regulation of cell death.
responsive to environmental stress: following 40Gy IR or heat shock, more cells in the imaginal discs are stimulated to exhibit increased DsRed signal, suggesting IRER is open in a larger range. Our data indicated that epigenetic regulation plays a significant role in determining the cellular sensitivity to stress induced cell death.

2122/B501  
**Isoform- and Dose-Sensitive Regulation of δ-Catenin Expression and Apoptosis by Paired Box 6 Gene.**

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Pax6, a member of the paired box gene family expressed in stem and progenitor cells during embryogenesis, resides at the top of the genetic hierarchy in controlling morphogenesis of the eye and other ectoderm derived organs. While genetic studies have identified δ-catenin (CTNND2) as a prominent candidate target gene, it is not clear how Pax6 regulates δ-catenin expression and functions. Here we report that overexpression of either Pax6(-5a) or Pax6(+5a) was sufficient to promote, whereas their knockdown reduced δ-catenin expression. Pax6(+5a) elicited stronger effects on δ-catenin than Pax6(-5a). Inducible Pax6(+5a) expression demonstrated a biphasic and dose-dependent regulation of δ-catenin expression and changes in cell shape. A moderate upregulation of Pax6(+5a) promoted δ-catenin expression and induced cellular protrusions, but increasing expression of Pax6(+5a) inhibited δ-catenin expression. Furthermore, sustained high expression of Pax6(+5a) triggered apoptosis involving Bcl-2 family, caspase, poly(ADP-ribose) polymerase, and survivin, while forced δ-catenin overexpression suppressed Pax6(+5a) induced apoptosis. Therefore, regulation of δ-catenin expression by Pax6 is not only isoform- and dose-dependent, but also provides important feedback mechanisms of their functional interactions in cellular morphogenesis, apoptosis and cancer.

2123/B502  
**Multiplexing Cell Proliferation and Cytotoxicity Assays Using Calcein Red and Cytocalceins.**

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Calcein AM is one of the most popular fluorescent probes used for labeling and monitoring cellular functions of live cells. However, the limited color selection of Calcein AM and its complete spectral overlap with GFP makes it impossible to use this valuable reagent in the multicolor analysis of live cells. Calcein Red and Cytocalceins have been developed for multiplexing analysis of cellular functions. The non-fluorescent Calcein Red and Cytocalceins become strongly fluorescent upon entering live cells. Calcein Red and and Cytocalceins are hydrophobic compounds that easily permeates intact live cells and generates their respective cell-retained fluorophores upon intracellular esterase-induced hydrolysis. The cellular esterase activity is proportional to the number of viable cells, and therefore directly related to the fluorescence intensity of Calcein Red and Cytocalceins. Using Calcein Red and Cytocalceins cell proliferation and cell cytotoxicity were monitored in HEK, CPA, and Jurkat cells. The consistent results were obtained with flow cytometry, fluorescence plate reader and florescence microscopy. Calcein Red and Cytocalceins have minimal cytotoxicity even after 2 weeks dye-loading in Jurkat cells while all the cells are apoptotic/necrotic after 3 days dye-loading with the same concentration of Calcein AM.
2124/B503

**DJ-1 Modulates the p38 Mitogen-Activated Protein Kinase Pathway through Physical Interaction with Apoptosis Signal-Regulating Kinase 1.**

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DJ-1 has been reported as a gene linked to early onset familial Parkinson’s disease, and is functionally involved in transcriptional regulation and oxidative stress-induced cell death. To understand the role of DJ-1 in cellular stress, this study investigated DJ-1’s effect on stress-activated protein kinase signaling and H2O2-induced activation of apoptosis signal-regulating kinase 1 (ASK1). According to the results, the overexpression of DJ-1 inhibited H2O2-induced activation of ASK1 as well as the activation of downstream kinases in the p38 mitogen-activated protein kinase (MAPK) signaling cascade. The results of both In Vivo binding and kinase studies have revealed that ASK1 is the direct target of DJ-1, whereas it has shown no effect on either M KK3 or p38. DJ-1 blocked both the homo-oligomerization of ASK1 and inhibited ASK1 activity. Taken together, our data strongly suggest that DJ-1, by directly inhibiting ASK1, may act as a negative regulator in ASK1 signaling cascades.

2125/B504

**Mechanism of Action of Atorvastatin in the Induction of Cell Death.**

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Objectives. Atorvastatin belongs to a class of drug known as statins, which are competitive inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, a rate-limiting enzyme in the cholesterol biosynthesis pathway. They also prevent the synthesis of downstream products which are needed for prenylation of many small regulatory GTPases involved in the regulation of cell cycle progression. Hence, statins have an unexpected effect of inhibiting growth of a number of cancer cells, but their mechanism of action is still not well established. The objective of this research project is to establish the mechanism of action of atorvastatin in the induction of apoptosis in cancer cells. Methods. Human prostate cancer cell line, PC-3, was used as a model to study the mechanism of action of atorvastatin. Cell growth and death were determined using CellTiter and LDH-based cytotoxicity assays, respectively. Initial survey of pathway(s) by which atorvastatin acts was done using various protein kinase inhibitors, antioxidants and other pathway-specific agents. Activation of specific gene was determined by promoter-luciferase reporter assays, and confirmed by RT-PCR. Results. Atorvastatin was found to induce death in PC-3 cells at a concentration of 100 nM. Initial survey of possible pathway using various pathway-specific inhibitors or agents showed that only geranylgeranyl pyrophosphate was able to protect the cells from atorvastatin-induced cell death, suggesting prenylation of the GTPases plays an important role. on the other hand, luciferase reporter assays demonstrated the activation of ERSE and AP-1 response elements of the CHOP promoter, and p53 and SP-1 response elements of p21 promoter. Activation of these two genes was confirmed by RT-PCR. Conclusions. Atorvastatin induces cell death in PC-3 cells using multiple pathways. The major pathway involves blocking the prenylation of small GTPases by preventing the synthesis of geranylgeranyl pyrophosphate. In addition, it also induces the Unfolded Protein Response by inducing CHOP, a gene involves in ER stress. It also induces cell growth arrest by up-regulation of cell cycle regulatory protein, p21.

2126/B505

**Modulation of Bax by Mammalian Protein Kinase Cα in Yeast.**

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Protein kinase C (PKC) is a family of serine/threonine kinases with at least 10 isoforms grouped into 3 subfamilies based on their primary structure and cofactors required for activation: classical,
novel and atypical. PKC isoforms are important regulators of several cellular processes, such as cell proliferation and death. Due to their role in the regulation of major apoptotic members, namely Bcl-2 family proteins, their function in cell death have been extensively investigated. Yet, the role displayed by each PKC isoform in cell death is controversial, essentially due to the co-existence of several isoforms in same cell. Yeast lacks orthologs of the Bcl-2 family proteins and, though yeast has a PKC ortholog, the mammalian PKCs do not functional complement this kinase. Yet, when these mammalian proteins are expressed in yeast, they conserve their functional and molecular properties. Recently, we showed that distinct PKC isoforms can differently modulate Bcl-xL anti-apoptotic effect in yeast (Saraiva et al. (2006) J Cell Sci 119:3171-3181). This work supported our purpose to use yeast as a model system to study the regulation of other Bcl-2 family members, such as Bax, by PKC isoforms. The first isoform studied was the classical PKCα. The role of PKCα in Bax regulation was studied using yeast co-expressing these mammalian proteins. Two forms of Bax were co-expressed with PKCα, Bax c-myc (an active form of Bax) and native Bax. We observed that PKCα co-expression increases Bax c-myc-induced cell death, an effect accompanied by enhancement of reactive oxygen species production, mitochondrial network fragmentation and Bax c-myc translocation to mitochondria. Additionally, we showed that in yeast Bax c-myc is not phosphorylated and that PKCα does not alter Bax c-myc phosphorylation state. Consistently, treatment with typical PKC inhibitors had no effect in the enhancement of cell death, and co-expression with a non-phosphorylating PKCα had the same effect as normal PKC. In contrast, native Bax is phosphorylated in yeast and co-expression of PKCα leads to its dephosphorylation. Our results reveal that PKCα modulates Bax and validate yeast as a tool to study regulation of Bax by PKC isoforms.

2127/B506
Apoptosis Induction via p38-Mediated AP-1 Activation Against B16/F10 Melanoma Cells by Lycoris Radiata Extract.

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Some active alkyloids isolated from Lycoris, a bulbous perennial herb, has shown to possess various anti-tumor and anti-inflammatory activities. In this study, we evaluated the In Vitro apoptotic effect of ethanol extract from Lycoris radiata (LRE) and further probed the underlying molecular mechanisms of any LRE effects. The survival rate of B16F10 melanoma cells exposed to LRE was decreased in a dose-dependent manner, cell growth was retarded by arresting cell cycle at G1 phase, and apoptotic appearances such as caspase-3 activation as well as DNA fragmentation were observed by LRE treatment. In addition, LRE induced p38 and c-Jun phosphorylation, followed by activation of transcription factor AP-1. Pretreatment with the p38 inhibitor (SB203580) blocked LRE-induced AP-1 transcriptional activity, and curcumin, AP-1 inhibitor, dramatically inhibited LRE-induced apoptosis in B16F10 melanoma cells. Our results collectively indicate that LRE-mediated apoptosis occurs through the activation of p38 and AP-1 pathway, and LRE exhibits a potential anti-cancer activity against B16F10 melanoma cells.

2128/B507
Elucidating the Pleiotropic Effects of SK1-I, a Novel Sphingosine Kinase 1 Inhibitor, Using a Battery of Cell-Based Assays.

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The potent bioactive sphingolipid mediator, sphingosine-1-phosphate (S1P), is produced by two sphingosine kinase isoenzymes, SphK1 and SphK2. Expression of SphK1 is upregulated in cancers, including leukemia, and is associated with cancer progression. A screen of sphingosine analogs identified SK1-I (BML-258), as a potent, water soluble, isozyme-specific inhibitor of SphK1. In contrast to pan-SphK inhibitors, SK1-I does not inhibit SphK2, PKC, or numerous other
protein kinases. SK1-I has previously been shown to decrease growth and survival of human leukemia U937 and Jurkat cells, and enhance apoptosis and cleavage of Bcl-2 (Blood (2008) 112(4):1382-91). We further evaluated SK1-I using a battery of cell-based assays. Addition of SK1-I leads to a rapid mobilization of intracellular calcium stores, a decrease in mitochondrial membrane potential, exposure of phosphatidylserine on the extracellular face of the plasma membrane, plasma membrane leakiness to a cell-impermeant DNA-intercalating dye and nuclear condensation, all consistent with its reported action as a pro-apoptotic agent. In addition, SK1-I was found to induce phospholipidosis in mammalian cell lines. Phospholipidosis was observed in U-2-OS osteosarcoma cells, treated for 19 hours with SK1-I at a half maximal effective concentration (EC50) value of 3.5 µM. Previous studies have shown that in U937 cells, SK1-I causes an increase in ceramide lipid accumulation, with a decrease in sphingomyelin species. The abnormal accumulation of ceramide may lead to the phospholipidosis response. Our studies confirm the potent activity of SK1-I as an apoptosis-inducing agent and provide further insight into the consequences of S1P inhibition on overall lipid metabolism.

2129/B508
Normal Human Fibroblasts Exhibit Diminished Caspase 8 and c-Myc Protein Expression and Are Resistant to TRAIL-Mediated Apoptosis.
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TNF-related apoptosis-inducing ligand (TRAIL)-mediated cell death offers an exciting therapeutic opportunity in cancer. However, the molecular basis of normal cell resistance to TRAIL-mediated apoptosis, and thus therapeutic window, remains largely unexplored. We found that normal HFF and WI38 fibroblasts expressed slightly lower levels of TRAIL receptor DR5 as compared to human lung and colon cancer cells. While normal and cancer cells showed comparable DcR2 expression, the WI38 and HFF expressed little DR4 and DcR1. Normal fibroblasts were examined for increased expression of proteins known to inhibit TRAIL-mediated cell death. Anti-apoptotic Bcl-xL and Mcl-1 expression in WI38 and HFF was comparable to expression found in cancer cells, while no XIAP expression was detected in the normal fibroblasts. c-Myc, a major determinant of TRAIL sensitivity in cancer cells, was found decreased in the normal fibroblasts and thus may be a contributing factor to their escape from TRAIL-induced cell death. NF-κB activation was assessed after 4 hours of TRAIL exposure, but minimal IκB degradation was observed in the normal fibroblasts, suggesting their resistance to TRAIL may not be mediated through induction of pro-survival factors by NFκB. Expression and activation of initiator caspases, required for complete TRAIL signaling, was determined. Although its levels were elevated, no caspase 10 activation was observed in normal WI38 and HFF cells after TRAIL treatment. Minimal caspase 8 protein expression was detected in the normal human fibroblasts, at levels barely detectable as compared to tumor cells that generally show robust caspase 8 expression. Reduced caspase 8 expression may be another contributing factor to the resistance of normal cells to TRAIL. Our current studies are focused on further analysis of mediators of cell death signaling by TRAIL, whether treatment of normal fibroblasts with proteasome inhibitors, histone deacetylase inhibitors, and/or DNA methylation inhibitors might result in increased caspase 8 protein expression and susceptibility to TRAIL-mediated apoptosis.

2130/B509
PA28γ Regulates Ultraviolet Radiation Induced Apoptosis Independently of JNK MAPK.
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The ability of PA28γ to control both the cell cycle and apoptosis has important implications in understanding cancer and developing novel treatments. Proteasome activator PA28γ is an ubiquitin- and ATP-independent activator of 20S proteasomes that has been proposed to control apoptosis and the cell cycle by degrading specific proteins such as p53 and p21Cip1. While PA28γ is known to degrade p53, the complex phenotype displayed by PA28γ-/- cells likely involves more
than one level of apoptotic regulation. While PA28γKO (KO) cells display increased levels of spontaneous apoptosis, the KO cells are also resistant to the effects of several apoptotic inducers. The c-Jun N-Terminal Kinase (JNK) pathway is one of the most common pathways leading to apoptosis and thus a potential target for PA28γ. Under normal cellular conditions, there is no significant difference in JNK1 or JNK2 concentrations in either wild type (WT) or KO cells, nor was there a difference in the half-life of either protein. The JNK inhibitor, SP-600125, does not differentially affect UV-induced apoptosis in the WT or KO cells, while dramatically reducing overall levels of apoptosis in both cell lines. However, both JNK1 and JNK2 appear to be stabilized and Bax levels increase in the KO cells following treatment with UVR. While a role for PA28γ in the JNK pathway seems unlikely under basal conditions, PA28γ may be working to counteract JNK signaling when the cell is undergoing apoptosis or stress.

2131/B510
Mitochondrial p38MAPKinase Regulates Bax Activation during Anoikis.
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The crucial function of apoptosis for metazoan survival is highlighted by the broad spectrum of diseases associated with abnormal levels of cell death. In most cells, the critical 'point of no return' in apoptosis signalling is mitochondrial outer membrane permeabilisation (MOMP), which is mediated by the pro-apoptotic protein Bax. Bax activation occurs in distinct stages prior to MOMP; an initial, reversible translocation to the mitochondria is followed by an irreversible activation, concomitant with cytochrome c release. Using a physiologically relevant apoptosis model we have shown that mitochondrial p38MAPK provides the signal responsible for the second step of Bax activation, which commits cells to death. Anoikis is a form of apoptosis induced by the loss of extracellular matrix (ECM)-dependent survival signals. In a non-transformed mammary epithelial cell line we identified that p38MAPK was activated during anoikis. Inhibition of p38MAPK blocked anoikis by preventing activation of Bax on the mitochondria, but without affecting Bax translocation. p38MAPK translocated to the mitochondria during anoikis and formed a membrane-associated high molecular weight complex, which implied a mitochondrial scaffold protein was involved in recruiting p38MAPK. Targeting p38MAPK to the mitochondria increased cell death in response to ECM-withdrawal. Moreover, cells committed to undergoing apoptosis sooner when mitochondrial p38MAPK was activated by co-expression with active MAPKinase Kinase 6. Our findings demonstrate that p38MAPK has a key role to regulate Bax activation on the mitochondria during anoikis. As resistance to anoikis is a feature of cancer cell metastasis, further characterisation of the role p38MAPK plays during anoikis may provide novel therapeutic targets for cancer treatment.

Meiosis and Mitosis III (2132 – 2158)

2132/B511
A Kinesin-Like Protein That Caps Microtubule Plus Ends.
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Equatorial chromosome positioning promotes proper chromosome segregation by increasing the chance that sister chromatids will be located at opposite poles during anaphase. The human Kinesin-8 Kif18A, a motor that localizes to plus ends of kinetochore-microtubules, is essential for metaphase chromosome alignment yet how it contributes to chromosome motility remains unclear. Rates of chromosome movements in Kif18A-depleted cells are elevated, suggesting that Kif18A might stabilize kinetochore-microtubule plus ends rather than depolymerizing them as reported for Kip3, its yeast orthologue. By studying the In Vitro properties of Kif18A, we found that Kif18A is a moderately fast (vmax=−8 μm/min) plus end-directed motor. Upon translocation to
plus ends, Kif18A does not induce microtubule disassembly but rather prevents polymerization through a capping-like mechanism. To explore if Kif18A similarly affects microtubule dynamics in vivo, we mapped the Kif18A nuclear localization sequence (NLS) and engineered a mutant protein, Kif18A-NLSmut, which is not retained in the nucleus during interphase. Consistent with a capping-like activity, Kif18A-NLSmut localizes to tips of interphase microtubules, and blocks microtubule assembly in re-growth assays. Quantitative analysis of interphase microtubule dynamics demonstrates that Kif18A-NLSmut causes microtubules to pause frequently, where filaments neither grow nor shrink. Our results suggest that Kif18A does not affect kinetochore-microtubule dynamics by catalyzing their depolymerization, but rather acts by capping their plus ends and dampening their dynamics.

2133/B512
Kinesin-14 Cross-Species Analysis Reveals Shared and Distinct Roles in Spindle Bipolarity.
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In eukaryotes formation of a conserved mitotic spindle apparatus enhances genomic fidelity and is dependent on kinesin-like motor proteins (Klps) for its assembly, maintenance and function. Ubiquitous Kinesin-5 promoting and Kinesin-14 opposing forces balance to regulate spindle assembly and stability. Different Kinesin-14 roles in bipolarity are suggested by γ-tubulin-based microtubule organizing center interactions at poles that influence establishment in fission yeast and by microtubule cross-linking and sliding that maintain bipolarity and spindle length in Drosophila. Cross-species analysis was performed to test functional compatibility of Kinesin-14 members in spindle establishment. The ability of human HSET (HsHSET) and Drosophila Ncd (DmNcd) in replace Schizosaccharomyces pombe Pkl1 (SpPkl1) was examined in vivo. HsHSET replaces SpPkl1 and similar modes of action are suggested by the ability to block HsHSET function by mutation in a defined Kinesin-14 binding site on γ-tubulin. DmNcd does not replace SpPkl1. It localizes preferentially to bundled interpolar spindle microtubules, unlike the more uniform spindle and additional pole localization of HsHSET and SpPkl1. By In Vivo analysis of thirty Kinesin-14 derivatives, including Tail, Stalk or Neck-Motor chimeras, for spindle assembly, spindle localization and mitotic progression we defined domains critical for spindle establishment. SpPkl1 functions effectively with the DmNcd Neck-Motor domain or by complete replacement of its Stalk with the leucine zipper sequence from budding yeast transcription factor GCN4. SpPkl1 Tail elements are distinct from those of DmNcd and were defined by extensive mutational analysis and conservation with related genus member, S. octosporus. Replacement of the DmNcd Tail with that of SpPkl1 was necessary to allow its cross-species function. Flexibility in the design plan of Kinesin-14s, in part through varying Tail elements, broadens their mechanistic possibilities in eukaryotes that include distinct roles in spindle assembly and maintenance. Additional mitotic Klp families with observed roles in spindle assembly must contribute to fill vacant functional roles occurring as a result of altered Kinesin-14 design.

2134/B513
Characterization of a Novel Interaction between Myosin-10 and a Mitotic Kinase.
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The unconventional myosin, myosin-10 (myo10), contributes to the structural integrity and/or function of various cellular structures including filopodia and meiotic and mitotic spindles. Like all myosins, myo10 is comprised of an amino-terminal head domain that binds actin and hydrolyzes ATP, and a carboxy-terminal tail domain. The tail of myo10 harbors multiple functional domains, which facilitate interactions with various macromolecules including microtubules, phospholipids and integral membrane proteins. Previous work from our lab revealed that myo10 localizes to the mitotic spindle and depletion of myo10 protein from Xenopus laevis embryos results in several mitotic spindle-related phenotypes including spindle elongation, failed anchoring, mitotic delay
and pole fragmentation. In order to identify novel myo10 interacting proteins that may contribute to its mitotic spindle-related functions, a yeast 2-hybrid screen was performed with a fragment of the myo10 tail, with a mitotic kinase scoring the most positive hits. Here we independently verify and further characterize this physical interaction and demonstrate a genetic interaction between myo10 and the mitotic kinase with respect to some of the spindle phenotypes. Together these results suggest that myo10 functions as a link between the cytoskeleton and mitotic progression. Lastly, using 4D imaging of myo10 fragments fused with GFP we provide surprising evidence that myo10's spindle pole localization does not require its microtubule-interaction domain.

2135/B514
Cdk1 and Polo-Like Kinase 1 Control the Temporal Activation of Haspin Kinase in M Phase.
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Coordination of multiple complex events during M phase requires the timely activation and inactivation of numerous mitotic protein kinases. The kinase Haspin is known to phosphorylate histone H3 threonine 3 (H3T3) during M phase, but the mechanistic basis for the regulation and function of Haspin remains unknown. We demonstrate that in Xenopus metaphase egg extract, two other M phase specific protein kinases, Polo-like kinase 1 (Plx1) and Cdk1-cyclin B, phosphorylate and collaboratively activate Haspin. In Plx1-depleted egg extract, histone H3T3 phosphorylation is greatly impaired, suggesting that Plx1 regulates Haspin. Substrate specificity of Plx1 is often ensured by docking of its phosphopeptide binding module, the polo box domain (PBD), to prephosphorylated targets comprising an S(pS/pT)(P/X) motif. The N terminus of Haspin contains an evolutionarily conserved STP motif, and we show that the prephosphorylation of this motif by Cdk1 promotes binding of purified Plx1-PBD to Haspin. Consistently, a point mutation of the Plx1-docking site abolishes Haspin-dependent phosphorylation of H3T3 in egg extracts. Our data strongly suggest that Haspin is under the tight control of Cdk1 and Plx1. We are currently investigating the functional significance of the timely activation and inactivation of Haspin during M phase.

2136/B515
Recycling of Connexin43 during Cell Division.
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Direct cell-cell communication via gap junctions (GJs) helps cells maintain uniform cytosolic metabolites and ion concentrations, however during the cell cycle the levels of GJ communication (GJC), morphology and distribution of connexin43 (Cx43)-containing structures change dramatically. As cells round up for mitosis, the majority of Cx43 labeling is intracellular and correlated with a reduction in GJC which can be rapidly resumed upon cytokinesis. The processes by which GJ plaques and cytoplasmic structures are disassembled and reassembled during mitosis are not clear, nor are the signaling pathways involved. Objective: we investigated the fate of Cx43 during mitosis both in endogenous and exogenous expressing cells using optical pulse-chase labeling and correlated light and electron microscopy (EM) in combination with immunocytochemistry and biochemical analysis. Results: time-lapse imaging of Cx43-GFP-4C expressing cells revealed an early disappearance of GJ plaques, progressive accumulation of Cx43 in cytoplasmic structures and a persistent pool of protein concentrated in the plasma membrane surrounding the midbody region in telophase. These distributions were also observed in endogenous Cx43-expressing NRK and RAT1 cells. To explore the origin and cellular localization of Cx43 during cell division we synchronized MDCK cells stably expressing Cx43 309/337 4C in G1/S then released them in order to perform pulse-chase labeling with the spectrally distinct ReAsH-EDT2 and FlAsH-EDT2 as cells progressed towards and through
mitosis. This method allows spatial and temporal discrimination of Cx43 species synthesized before and through mitosis. In late telophase, older Cx43 is localized at the plasma membrane, as apparent hemichannels while newer Cx43 is intracellular. An antibody specific for Cx43 phosphorylated on S279/282 (MAPK substrates involved in GJ downregulation) recognized the older Cx43 species. Photo-oxidation of Cx43-GFP-4C cells in telophase and high resolution EM confirms that this older Cx43 pool is in the plasma membrane surrounding the midbody. Conclusions: older Cx43 may act as nucleating sites for new formation of GJ plaques as they redistribute in the plasma membrane of the daughter cells.

2137/B516
Mechanism of Activation of the Greatwall Kinase in Cell Cycle.
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Greatwall (GW) is a novel protein kinase that plays an important role in mitotic entry and maintenance. Depletion of GW from metaphase II-arrested Xenopus oocytes induces mitotic exit, whereas the same depletion in cycling extracts prevents mitotic entry. GW is phosphorylated at mitotic entry and dephosphorylated at exit of mitosis and this phosphorylation is concomitant with GW activation. Nothing is known about the kinase/s that are responsible for this activation. Taking into account that this kinase is essential to induce a correct timing of mitosis, the characterization of the mechanisms controlling this activation is crucial. In this study we combine a mutagenesis and a phosphomapping analysis to identified the different phosphorylation sites of GW at mitotic entry. We also analyse the activity of the different mutants in Xenopus egg extracts as well as the localization of these mutants in human cells and the phenotypes induced by their overexpression in human cells. Finally, we performed different “in vitro” and “in vivo” tests in order to identify which is/are the kinase/s responsible for these phosphorylations.

2138/B517
The Role of CLASP Phosphorylation during Mitosis.
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Correct distribution of the genetic information during cell division requires chromosome attachment to spindle microtubules via the kinetochore and subsequent segregation by the mitotic spindle. Since kinetochore microtubules push and pull chromosomes during metaphase chromosome alignment and anaphase chromosome segregation, the plus-ends of kinetochore microtubules must remain dynamic and kinetochore-microtubule attachment has to be precisely regulated. However, little is known on how the kinetochore-microtubule attachment is regulated. CLASPs are proteins that associate with growing microtubule plus ends, and are components of the kinetochore required for correct microtubule polymerization dynamics. Here, we have used confocal live cell microscopy to further investigate CLASP dynamics during mitosis. Surprisingly, although EGFP-tagged CLASP localizes to kinetochores throughout mitosis in HeLa cells, we found that CLASP disappears from microtubule plus-ends during prophase and reappears during late anaphase. This indicates that mitotic regulation of CLASP-microtubule association may be an important determinant of the regulation of kinetochore-microtubule association. We have recently found that CLASP is phosphorylated by glycogen synthase kinase 3 beta (GSK3β) on eight serine residues in the plus-end tracking domain (Kumar 2009), and that this phosphorylation regulates CLASP-microtubule association during interphase. By using non-phosphorylatable CLASP mutants, we now show that these same sites are highly phosphorylated during mitosis, and that phosphorylation disrupts CLASP-microtubule association in metaphase cells. HeLa cell lines in which CLASPs are depleted by RNAi exhibit characteristic spindle and chromosome segregation defects and we are currently investigating whether GSK3β-mediated CLASP phosphorylation is required for mitotic CLASP function.
**Ska3 Is Required for Mitotic Checkpoint Silencing.**

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The mitotic spindle checkpoint monitors proper bipolar attachment of chromosomes via their kinetochores to the mitotic spindle. Normally, the presence of stable microtubule-kinetochore interactions will relieve the checkpoint and permit the anaphase promoting complex/cyclosome (APC/C) to stimulate the degradation of mitotic effectors thereby initiating anaphase and triggering mitotic exit. The Spindle and Kinetochore Associated (Ska) proteins, Ska1 and Ska2, physically interact and are necessary for timely anaphase onset. Recently, we and others have characterized C13orf3/Ska3, a newly discovered component of the Ska complex. We found that Ska3 accumulates at kinetochores upon nuclear envelope breakdown and reaches maximal levels at metaphase. Upon anaphase onset kinetochore-associated Ska3 levels decline and are eventually lost coincident with nuclear membrane reassembly at telophase. We demonstrate that cells depleted of Ska3 by RNAi achieve metaphase alignment but fail to silence the spindle checkpoint and enter anaphase. During this mitotic arrest with chromosomes aligned at the metaphase plate, kinetochores retain robust microtubule attachments. However, kinetochores within these Ska3-depleted cells accumulate high levels of the checkpoint protein Bub1. In contrast, levels of the checkpoint proteins BubR1 and Mad2 at kinetochores of metaphase aligned chromosomes do not increase in Ska3 depleted cells. Cells arrested with chromosomes aligned at the metaphase plate by the proteasome inhibitor MG132 do not elevate levels of kinetochore-associated Bub1, BubR1, or Mad2. Therefore, Ska3 depletion induces elevated kinetochore levels of Bub1 which cannot be ascribed solely to an extended metaphase arrest. We hypothesize that Ska3 is required to down-regulate Bub1 activity in response to proper kinetochore-microtubule attachments thereby releasing the mitotic checkpoint and stimulating APC/C activity.

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**Analysis of Two Cdc20 Binding Domains in BubR1 Reveals Distinct Roles of Each BubR1-Cdc20 Interaction in Mitotic Checkpoint Function.**

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Mitotic checkpoint ensures accurate segregation of duplicated chromosomes into two daughter cells during mitosis by targeting Anaphase Promoting Complex or Cyclosome (APC/C), an E3 ubiquitin ligase, for inhibition. BubR1, a key molecule involved in this process, has been isolated in complex with Bub3, Cdc20, and Mad2 from cells and shown to inhibit Cdc20-activated APC/C activity in vitro, thereby proposed to be anaphase inhibitor. Our previous efforts to elucidate the mechanism of anaphase inhibitor production have demonstrated that BubR1 inhibits Cdc20-APC/C in both kinetochore-dependent, in which unattached kinetochores catalyze Mad2 priming of Cdc20 for its association with BubR1, and kinetochore-independent manners. The underlying molecular mechanism of BubR1 involvement in two distinct pathways and whether or how these two pathways work together during cell cycle remain to be elucidated. Using In Vitro reconstitution of mitotic checkpoint, we here demonstrate that kinetochore(or Mad2)-dependent inhibition of Cdc20-APC/C is mediated by BubR1 fragment which contains N-terminal Cdc20 binding domain, while BubR1 fragment containing internal Cdc20 binding domain, which exists only in higher eukaryotes, directs kinetochore(or Mad2)-independent pathway. Quantitative comparison reveals that Mad2-dependent inhibition of Cdc20, even without boost by unattached kinetochore, is more potent than Mad2-independent inhibition. In Vivo replacement of endogenous BubR1 by various recombinant BubR1 fragments elucidates that N-terminal Cdc20 binding domain provides with basic mitotic checkpoint function, while internal Cdc20 binding domain instead supports prolonged mitotic arrest of cells that are under nocodazole challenge. These results suggest that the additional internal Cdc20 binding domain in higher eukaryote
BubR1 allows cells more time to deal with complicated mitotic errors and thus contributes to preventing aneuploidy of the offspring.

2141/B520
Separating the Spindle, Checkpoint and Timer Functions of BubR1.
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BubR1 performs several roles during mitosis. It is essential for checkpoint-mediated inhibition of the APC/C, but it also promotes proper kinetochore-microtubule linkages. BubR1, like Mad2, also determine the basal mitotic timing of mitosis -- the minimum time that elapses between Nuclear Envelope Breakdown (NEB) and anaphase onset even when there are no unattached kinetochores. However, the interdependence of these functions is unclear. We have analyzed In Vivo the mitotic phenotypes in Drosophila of a "kinase-dead" (KD) bubR1 mutation and a mutation lacking the N-terminal KEN box. bubR1-KD individuals are viable, display little aneuploidy and have a robust checkpoint capable of delaying anaphase onset when chromosomes are improperly attached to the spindle. However, the chromosomes are slow to align on the metaphase plate, K-fibers are thinner, and spindle length is unstable. The bubR1-KEN mutant, in contrast, shows no defect in spindle function, but by classical criteria it is "checkpoint-dead", being unable to arrest cells with damaged spindles. Like the previously described mad2 null mutant, mitosis is normal in bubR1-KEN mutant flies and aneuploidy is low, despite the absence of checkpoint activity. Basal mitotic timing however is normal, indicating that the mutation has also separated the checkpoint function from the "timer" function. Flies of genotype bubR1-KEN mad2, lacking both components of the checkpoint pathway are also viable, fertile, and have no more aneuploidy than either mutant alone, despite significantly accelerating the average transit time from nuclear envelope breakdown to anaphase onset. Our study shows that we can substantially uncouple the checkpoint activity of BubR1 from its spindle function, confirms that reliable mitosis in Drosophila does not need the spindle checkpoint, and suggests that the kinase activity of BubR1 modulates microtubule capture and/or kinetics at kinetochores, but is relatively dispensable for normal checkpoint function in flies.

2142/B521
Molecular Analyses of Tip150-BubR1 Interaction in Mitosis.
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The microtubule cytoskeleton orchestrates the cellular dynamics and plasticity that underlies morphogenesis and cell division. A growing number of microtubule-associated proteins "congregate" at the plus end, and are categorized as +Tip proteins responsible for microtubule plus-end regulation. +Tip protein interactions are required for chromosomal segregation and cellular division. Our recent study demonstrates that Tip150 interacts with plus end binding proteins EB1 and MCAK In Vitro and In Vivo (EMBO Reports. 2009. 10:857). However, it is unclear how this protein is regulated, and the function of Tip150 in microtubule assembly. To delineate the molecular function of Tip150 and its regulation, we carried out biochemical kinase assay using a recombinant Tip150 protein incubated with a battery of active mitotic kinases, and revealed that the Tip150 protein is phosphorylated by BubR1 kinase. To characterize a physical interaction between these two proteins, we performed an In Vitro binding assay in which recombinant Tip150 binds BubR1. Our immunofluorescent microscopic analyses show that Tip150 is co-localizing to BubR1 at the plus-end of spindle microtubule. Currently, we are carrying out proteomic analysis of BubR1-mediated phosphorylation site(s) and evaluating the functional relevance of Tip150 phosphorylation in cell division control.
Regulation of Mitosis and Taxane Response by Daxx and Rassf1.

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Current theories suggest that mitotic proteins are essential for cellular response to taxanes, a widely-used family of chemotherapeutic compounds. We recently demonstrated that absence or depletion of protein Daxx increases cellular taxol (paclitaxel) resistance—a common trait of patients diagnosed with breast cancer and other malignancies. An increased occurrence of pro-metaphase in Daxx knockout mouse cells suggested a potential function of Daxx in mitosis and cell division; Daxx is also important for the proper timing of mitosis in human cells. Daxx interacting partner, Rassf1, is a cytoplasmic, microtubule-associated protein that is important for normal mitotic progression and cell division. In pro-metaphase, Daxx is released from nuclei and co-localizes and interacts with Rassf1 at mitotic spindles. Rassf1/Daxx depletion or expression of Daxx binding domain of Rassf1 increases taxol resistance in cells and elevates cyclin B stability. Mouse xenografts derived from Daxx- and Rassf1-depleted cells are resistant to taxol treatment; in breast cancer patients, the inverse correlation was observed between Daxx and clinical response to taxane-based chemotherapy. These data suggest that Daxx and Rassf1 define a mitotic stress checkpoint that enables cells to efficiently exit mitosis (and eventually die) when encountered with specific mitotic stress stimuli, including taxol. Thus, Daxx and Rassf1 may become useful predictive markers for the proper selection of patients for taxane chemotherapy.

Mechanism of Activation of the Chromosome Passenger Complex.

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The Xenopus chromosome passenger complex (CPC), composed of Aurora B and its regulatory subunits INCENP, Survivin and Dasra, ensures correct chromosome alignment and segregation during mitosis. The CPC regulates each chromosome autonomously, however, the mechanism is not well understood. Positive feedback can explain how a checkpoint signal generated from a single misaligned chromosome can be amplified to cause mitotic arrest. We have investigated the kinetics of recombinant CPC in vitro. In our study we have also included a known activator of Aurora B kinase, Monopolar spindle 1(MPS1), a mitotic dual-specificity kinase. MPS1 has been shown to phosphorylate Dasra in vitro. Lambda-phosphatase treated CPC lacks critical activation marks such as phosphorylation of the Aurora B T-loop and lacks kinase activity. When a small amount of active CPC is added, the phosphatase-treated CPC is activated, indicating requirement of intermolecular phosphorylation. The kinetics of activation suggests positive feedback. Additionally, we have seen that MPS1 immuno-precipitated from Xenopus egg extracts can also activate lambda-phosphatase treated CPC in vitro. These data suggest that there might be cross-talk between the two kinases and we think that both might play a role in activating the other. In conclusion, our study so far reveals that the activation of the CPC involves a ‘trans’ phosphorylation event and generates a positive feedback loop, which could greatly amplify the checkpoint signal from a single unaligned chromosome.

Structure-Function Studies of the RCC1-Like Molecule, TD-60, Reveals a Domain-Based Regulation of the Chromosomal Passenger Complex.

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The Chromosomal Passenger Complex (CPC) is a heterotetrameric complex consisting of Aurora B kinase and its regulatory subunits INCENP, Survivin and Borealin/Dasra. During cell division, the catalytic activity of the CPC promotes proper chromosome congression and segregation through regulation of kinetochore-microtubule attachments, spindle checkpoint signaling and the establishment of the cytokinetic furrow. TD-60 is a RCC1-like molecule that contributes to the activation of the Chromosomal Passenger Complex (CPC) during mitosis. To date, little is known about the regulation of TD-60 itself, particularly about how its structure regulates its function. We have generated domain-specific mutants and used them to tease out various functions of TD-60 during mitosis. The N-terminus of TD-60 inhibits CPC activity both In Vitro and in vivo. In Vitro kinase assays reveal that the N-terminus of TD-60 inhibits CPC activation as well as activity, presumably through several putative and pseudo-substrate sites. Using both depletion with rescue, and overexpression experiments in Xenopus mitotic extracts, we have observed loss of CPC activity with the addition of the N-terminus of TD-60. We propose that TD-60 acts as not only as a positive, but also as a negative regulator of the CPC. Furthermore, we have linked these functions to distinct domains of TD-60.

2146/B525

Human EB Proteins Link Mitosis, Cell Adhesion and Motility By Regulating MT Dynamics.
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EB proteins are members of a group of microtubule (MT) plus-end binding proteins known as +TIPs. Two members of the family (EB1 and EB3) can exist as heterodimers and regulate microtubule growth. However, the role that this complex plays in mitosis is still unknown. To address the roles of EB proteins during mitosis we used a shRNA approach to deplete cells of EB1, EB3 or both proteins in tandem (EB1/EB3). While EB1-depleted cells failed to align the spindle parallel to the substrate during early mitosis, they are still capable of adhering to the substrate after anaphase onset. This correlated with the observation that astral MTs were severely reduced in EB1- but not EB3-depleted cells. EB1-depleted cells were also less motile than control or EB3-depleted cells. on the other hand, EB3-depleted cells were capable of aligning their spindle parallel to the substrate during early mitosis but unable to maintain attachment to the substrate once cells entered anaphase. These cells were also more motile than control or EB1-depleted cells. Curiously, we found that there was an increase in EB3 comets close to the cell cortex when furrow ingression started suggesting that EB3 is mediating cell adhesion during the later stages of mitosis. When EB-depleted cells were filmed, their spindles presented some degree of tilting and “shaking” back and forth in the cytoplasm, which indicates that in some aspects they are partially redundant. When EB1/EB3 depleted cells were transfected with an RNAi-insensitive EB3 containing only the MT binding domain, which is sufficient to rescue normal microtubule dynamics but does not bind to known cargo proteins, these cells showed normal motility and a normal adhesion timing. These data indicate that these processes are strictly dependent on MT dynamics and not mediated by EB-binding cargo. Interestingly, when this form of EB3 was over-expressed we could also rescue the formation of astral MTs, although this did not occur when EB3 was expressed only at normal levels. Our results indicate that, although there is a partial redundancy between EB1 and EB3, they are able to intervene at different mitotic and post-mitotic stages by regulating spindle position and cell adhesion, respectively.

2147/B526

P90 Ribosomal S6 Kinase-2 Specifically Affects Kinetochore-Microtubule Attachment.
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The Ras/mitogen-activated protein kinase (MAPK) pathway regulates various cellular processes including gene expression, cell proliferation, and cell cycle. Here, we have examined the potential involvement of p90 ribosomal S6 kinase (RSK), the downstream kinase of Ras/MAPK pathway, in the regulation of mitotic progression. To this end, siRNAs targeting RSK1 and RSK2 isozymes were transfected to HeLa cells. Interestingly, depletion of RSK2, but not RSK1, resulted in the accumulation of mitotic cells compared with control cells. Time-lapse imaging using GFP-H2B cells revealed the prolongation of prometaphase and metaphase duration in RSK2-depleted cells. In addition, we observed that increase of metaphase cells with bipolar spindles containing unaligned chromosomes and weakening of chromosome congression in RSK2-depleted cells. Moreover, RSK2 depletion results in the shortening of interpolar distance and the loss of tension across sister kinetochores at metaphase. Analysis of cold-stable microtubules revealed a significant decrease of KT-MT attachment in RSK2-depleted cells. Weakening of the tension across sister kinetochores led to activation of spindle checkpoint, evidenced by the residual Mad2 and BubR1 proteins in the kinetochores of metaphase spread. In addition, knock-down of BubR1 abolished the increase of mitotic index induced by the depletion of RSK2, confirming the involvement of mitotic checkpoint. Taken together, these results suggest that RSK2 affects KT-MT attachment during mitotic progression.

2148/B527
Identification of a Novel Cell Cycle-Regulated Gene Involved in Maintaining Spindle Bipolarity.
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Successful segregation of chromosomes during mitosis requires the establishment of a bipolar spindle. Bipolar spindle formation relies on both microtubule dynamics and sliding, as depletion or inactivation of kinesin motor proteins Eg5 or Kif2A leads to monopolar spindles. To identify novel genes involved in spindle morphology, we utilized the gene expression profile of cycling HeLa cells and hierarchical clustering. Several uncharacterized genes were identified that show peak expression during G2/M phase and are co-expressed with genes known to function in establishing the mitotic spindle. Here, we use siRNA, epitope-tagging, fixed and live cell fluorescence imaging, and co-immunoprecipitations to examine the function of one candidate gene, Fam64A, in HeLa cells. Database queries for Fam64A show no known protein domains in the predicted protein sequence, and no sequence similarity to other human genes; the protein also has uncharacterized orthologs in placental mammals. FAM64A is cell cycle-regulated at multiple levels. The mRNA shows periodic expression during the cell cycle with peak expression in G2/M phase. FAM64A is also regulated by the proteasome, as the protein is degraded during G1 phase, which is inhibited by the addition of MG132. Depletion of FAM64A using three independent siRNAs results in a 10-fold increase in the number of mitotic cells exhibiting monopolar spindle. Expression of a non-siRNA targetable FAM64A-GFP construct results in a partial rescue of the monopolar defect, suggesting the phenotype is not an off-target effect. The FAM64A-GFP fusion protein localizes to the nucleus during interphase and to the cell cortex in mitosis. The inability to detect spindle localization suggests FAM64A contributes to spindle bipolarity through mechanisms that are independent of other motors like Eg5 and Kif2A.

2149/B528
The Proteasome Component Rpn5 Is a Nuclear Matrix Protein in Aspergillus nidulans and Its Inheritance in Mitosis Is Disrupted By γ-Tubulin Mutations.
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The phenotypes of a mutant γ-tubulin allele of Aspergillus nidulans have led us to investigate the effects of γ-tubulin mutants on localization of the proteasome. We have found that the proteasome subunit RPN5 is essential and have created functional RPN5-GFP and RPN5-mCherry fusions under the control of the endogenous RPN5 promoter. Imaging of RPN5 and
histone H1 (to visualize chromatin) revealed that in the wild type, RPN5 is concentrated in the nucleoplasm in interphase. At mitotic onset when the nuclear pore complex disassembles and soluble proteins diffuse into the cytoplasm, RPN5 remains in the nucleus. At anaphase it is located between separating chromatin masses. It disappears in telophase, but immediately begins to accumulate in daughter nuclei. In cells treated with the anti-microtubule agent benomyl and in a γ-tubulin deletant that prevents spindle formation and causes aberrant mitosis, single time point imaging revealed that some nuclei had a great deal of RPN5 and little chromatin, while others had a great deal of chromatin and little RPN5. Time-lapse imaging in the γ-tubulin deletant, and in a cold-sensitive γ-tubulin mutant, revealed that chromatin and RPN5 often separate from each other during aberrant mitosis resulting in chromatin and RPN5 occupying separate, although sometimes overlapping, portions of the nucleoplasm. The fact that RPN5 occupies only a portion of the nucleoplasm reveals that it (and by extension the proteasome) is not freely diffusible but must bind to something within the nucleus that is separate from chromatin but has structural integrity (i.e. a nuclear matrix). It follows that RPN5 fluorescent fusion proteins allow live imaging of this nuclear matrix and that the inheritance of the nuclear matrix is altered by γ-tubulin mutants, benomyl and, presumably, other mitotic disrupting agents. Deletion of the mlp1 gene, which has recently been reported to encode a protein (MLP1) that is required for localization of certain mitotic regulatory proteins to a spindle matrix in A. nidulans, did not alter the location of RPN5 so RPN5 does not bind to the spindle matrix through MLP1 although both may bind independently to the same matrix. Supported by grant GM031837 from the NIH.

2150/B529

Regulation of Protein Phosphatase 1 at the Kinetochore.

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Accurate chromosome segregation in mitosis is necessary to ensure that each daughter cell gets a full complement of the genome. This process depends on correct attachment of microtubules to the kinetochore. The phosphorylation state of several kinetochore proteins regulates this attachment, which reflects a balance between kinase and phosphatase activity. For example, it has been proposed that erroneous kinetochore-microtubule attachment is corrected by the kinase Aurora B at inner centromeres. Protein Phosphatase 1 (PP1) also localizes at kinetochores where it is thought to antagonize Aurora B. However, how PP1 is targeted and regulated at the kinetochores is still unknown. We are examining this question biochemically using the Xenopus egg extract system. The catalytic core of PP1 binds to a variety of regulatory proteins that contain an RVxF motif. To test whether PP1-kinetochore targeting is mediated by one of these regulators, we created a PP1 mutant that fails to bind the RVxF motif. The RVxF-binding mutant of PP1 did not fully enrich at kinetochores, indicating that a kinetochore protein containing an RVxF motif contributes to PP1 targeting. Using a candidate approach and non-biased database searching, we identified the kinetochore protein Blinkin (also known as Knl1 and CACS5) as a potential targeting protein, which has an evolutionarily conserved RVxF motif in the N-terminus. Blinkin has recently been described to interact with BubR1 and Bub1, and this interaction has been suggested to modulate the spindle assembly checkpoint and microtubule attachment functions of these kinases. We have shown that the N-terminus of Blinkin interacts with PP1 in an RVxF dependent manner, and this interaction is sensitive to okadaic acid. These data suggest that Blinkin targets PP1 to the kinetochore, and that this targeting may be regulated by a phosphorylation event. We are currently exploring how the phosphorylation of Blinkin may modulate the function of PP1 at the kinetochore.

2151/B530

Cofilin Localizes to the Centrosome and Is Required for Microtubule Anchoring and Mitotic Bipolar Spindle Assembly.

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The actin dynamizing protein cofilin is essential for diverse cellular processes dependent on dynamic actin cytoskeletal remodeling, such as cell division. Here we show that cofilin localizes to the centrosomes of mammalian cells, independently of microtubules, throughout the cell cycle. At the centrosome, cofilin’s phosphorylation state is modulated in a cell cycle dependent manner suggesting precise regulated control of cofilin activity at this structure. RNAi mediated knockdown of cofilin disrupts microtubule organization in both interphase and mitotic cells. In interphase cells cofilin knockdown disrupts the focused radial array of microtubules. This anomaly is associated with impairment of centrosomal microtubule anchoring but not microtubule nucleation. In mitosis, cofilin knockdown disrupts the organization of astral microtubules and mitotic spindles. Additionally, cofilin silencing results in a mitotic delay and the accumulation of cells in prometaphase with monopolar spindles. Time lapse analyses revealed that monopolar spindle defects resulted from impaired centrosome separation in cofilin knockdown cells. Together, our results identify roles for cofilin at the mammalian centrosome and establish new requirements for cofilin during mitosis. Our studies also suggest mechanisms by which cofilin may act to influence both the actin and microtubule cytoskeletons.

2152/B531
Relative Contributions of Chromatin and Kinetochores to Mitotic Spindle Assembly.
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During mitosis and meiosis in animal cells, chromosomes actively participate in spindle assembly by generating a gradient of RanGTP. A high concentration of RanGTP promotes microtubule nucleation and stabilization in the vicinity of chromosomes. However, the relative contributions of chromosome arms and centromeres/kinetochores in this process are not known. Here, we address this issue using human cells undergoing mitosis with unreplicated genomes (MUG). MUG occurs after prolonged S phase arrest in hydroxyurea and is characterized by assembly of a normal bipolar spindle, unreplicated kinetochores, and chromatin with a granular appearance. Nocodazole washout experiments and laser ablation of centrosomes demonstrate that both centrosomal and non-centrosomal spindle assembly pathways are active during MUG. Following nuclear envelope breakdown, the chromatin is rapidly separated from the forming spindle and kinetochores, presenting a unique opportunity to reveal specific roles for these components in microtubule organization. MUG chromatin is coated with the RanGEF RCC1 and establishes a RanGTP gradient which we visualized with FRET. Yet, the centrosomes and spindle are consistently centered at kinetochores/centromeres that are positioned well outside the peak concentration of RanGTP. Although chromatin is excluded from the spindle, it is connected to the poles through a subset of astral microtubules which are likely attracted to the RanGTP-activated microtubule stabilization factors. Finally, we test whether reducing kinetochore-microtubule interactions shifts the balance of spindle assembly toward chromatin. To this end, the outer kinetochore protein Nuf2 was depleted from both MUG and normal mitosis using siRNA. In depleted cells, chromatin continues to attract astral microtubules, but a spindle fails to form between separated centrosomes. This indicates that chromatin cannot support spindle assembly in mammalian cells even with the benefit of two centrosomes. This is different from the situation in Xenopus egg extracts where chromatin alone is sufficient. These results support a model in which kinetochores play a dominant role in the chromosome-mediated pathway of mitotic spindle assembly.

2153/B532
The Microcephaly Protein CDK5RAP2 Provides a Critical Link Between the Centrosome and the Mitotic Spindle.
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CDK5RAP2, a highly conserved centrosomal protein, is frequently mutated in primary microcephaly, a neurodevelopmental disorder characterised by reduced brain size. Since all known microcephaly gene products are centrosome or spindle pole proteins, it has been postulated that centrosome dysfunction underlies abnormal neural development. Here, we show that gene disruption of cdk5rap2 in the avian B-cell line, DT40, leads to premature centrosome separation in interphase and centrosome detachment from the spindle poles during mitosis. Mutant cells are viable in an exponentially growing culture, but their clonogenic potential is severely impaired. Real-time imaging reveals both an increase in cell death and a mitotic delay that closely correlates with centrosome detachment. Importantly, despite centrosome detachment, mitotic spindles remain bipolar and their poles focus normally in mutant cells. Our results indicate that microtubule-dependent forces are at least partially responsible for centrosome detachment. Since the ultrastructure of CDK5RAP2-deficient mitotic centrosomes appears intact, CDK5RAP2 could specifically mediate a subset of proteins required for microtubule tethering. We therefore propose that by safeguarding the integrity of the mitotic centrosome against microtubule-dependent forces, CDK5RAP2 connects centrosomes to spindle poles. Loss of connectivity between centrosomes and spindles could impede the regulation of asymmetric cell division of neural progenitors thereby leading to microcephaly.

2154/B533
Kinetochore-Microtubule Interactions Visualized By Electron Tomography.
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Recent structural studies have revealed differences between the plus-ends of microtubules (MTs) In Vivo and in vitro. Purified tubulin polymerizing In Vitro forms MTs whose ends comprise almost straight protofilaments (PF), but PFs on depolymerizing MTs are markedly curved. This distinction is not found in cells, where most PFs are curved, although the magnitude and length of PF curvatures vary (Cell 135: 322-33, 2008; Mol Biol Cell 19: 3138-46, 2008; J Struct Biol 161: 459-68, 2008). Here we use electron tomography to seek structural factors that might explain the differences between MTs In Vivo and In Vitro by looking at kinetochore-associated MTs in a range of organisms. PF curvatures in KMTs from the nematode C. elegans, the alga, Chlamydomonas, and the fission yeast, S. pombe are all rather similar to each other and to those previously described in mammalian cells, but PFs on KMTs from the budding yeast, S. cerevisiae are different. We found little significant difference between KMTs in these species at different stages of mitosis. All species studied showed evidence for fibrils that connected the bending PFs with nearby chromatin; these structures may correspond to mechanical linkages that alter PF structure. Two features of budding yeast KMTs distinguish them from their counterparts in other organisms: 1) in S. cerevisiae there are partial rings around most KMTs near their plus ends, and 2) the structure likely to correspond to centromeric chromatin appears as a small disk with a dark-staining rim. It looks like a nucleosome that is connected by fibrils to the bending PFs. These observations support the idea that filamentous couplers between chromatin and KMTs are a component of kinetochore-MT interactions in a wide range of organisms. They draw attention to factors that interact directly with the very ends of MTs, rather than their lateral walls.

2155/B534
Dynamics of Checkpoint Protein Complexes during Resolution of the Spindle Assembly Checkpoint.
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The spindle assembly checkpoint (SAC) functions to ensure that chromosomes are not segregated until they all form bipolar attachments, via kinetochores, to the mitotic spindle. The target of the SAC is the anaphase-promoting complex/cyclosome (APC/C) that is inhibited from degrading securin and cyclin B while kinetochores remain unattached. The checkpoint proteins, Mad2, BubR1, and Bub3 have been shown to form a complex known as the mitotic checkpoint complex (MCC) with Cdc20 that acts to inhibit the APC/C in mitotic cells. MCC-APC/C interactions must be tight during checkpoint activation but dissociate rapidly upon complete kinetochore attachment to permit timely anaphase onset indicating a complex regulation of MCC-APC/C affinity. We have investigated the interactions of MCC complex members, p31comet (a negative regulator of the SAC) and the APC/C to understand the dynamics of APC/C activation during SAC resolution. We have developed an assay, based on tandem affinity purification, to interrogate the assembly state of checkpoint complexes as well as phosphorylation state of proteins within that complex during SAC resolution. We find that Mad2 and BubR1 are both strongly associated with the inhibited APC/C and dissociate with kinetics that coincide with the onset of cyclin B degradation. Moreover, while Mad2 dissociates from Cdc20 during SAC resolution, a significant fraction of BubR1 remains bound to Cdc20 throughout mitotic exit. Finally we have investigated the interactions of p31comet with checkpoint complexes and find that this interaction is dynamic during mitotic exit. Together these data provide a timeline for protein association and dissociation that regulate the transition to anaphase. We are now using these biochemical finding to drive fluorescence cross-correlation measurements to verify these interactions and quantify their dynamics in living cells undergoing mitosis.

2156/B535

The CH Domains of Nuf2 and Hec1 Have Functionally Distinct Roles in Kinetochore-Microtubule Attachment.
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Successful mitotic cell division requires that end-on attachments be made between the kinetochore of each chromosome and the plus-ends of spindle microtubules. The kinetochore facilitates chromosome movement during mitosis by harnessing the energy of shortening and growing microtubule plus-ends. Critical for generating these functional attachment sites is the Ndc80 complex, a hetero-tetrameric complex comprised of Ndc80 (Hec1 in humans), Nuf2, Spc24, and Spc25. The Ndc80 complex is ~55 nm long, with globular heads on each end of a long, coiled-coil domain, potentially spanning the distance between the inner and outer kinetochore. In all cell types that have been studied, interference with the Ndc80 complex perturbs microtubule attachment to the kinetochore. Recent studies have demonstrated that the N-terminal globular domains of Nuf2 and Hec1 fold into a pair of calponin homology (CH) domains, which have been implicated in microtubule binding in vitro. The CH domains of both Nuf2 and Hec1 have positively charged ridges which have been proposed to make direct contact with the negatively charged, C-terminal tails of the microtubule lattice. Although progress has been made in defining the function of Hec1 in microtubule attachment, the function of Nuf2 at the outer kinetochore and in microtubule attachment remains unexplored. To characterize the role of Nuf2 at the kinetochore-microtubule interface, we used an siRNA-mediated silence and rescue approach in cultured cells. Endogenous Nuf2 was depleted from HeLa cells, followed by expression of either wild-type or mutant Nuf2-GFP fusion proteins. Here we show that the CH domain of Nuf2 is required to generate functional kinetochore-microtubule attachments, but that the surface charge composition of the CH domain of Nuf2 is not. In contrast, the surface charge of the Hec1 CH domain is critical for generating kinetochore-microtubule attachments. These data suggest that the CH domains of Nuf2 and Hec1 have distinct functions at the outer kinetochore.

2157/B535a

A Critical and Specific Role of Integrin Linked Kinase (ILK) In Centrosome Clustering in Cancer Cells.
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Cancer cells contain abnormal numbers of centrosomes, but are able to cluster them to avoid potentially lethal multi-polar mitosis. Molecules that regulate centrosome clustering in cancer cells could be potential tumor-specific cancer therapeutic targets (Cancer Cell, 14, 197, 2008). Here we demonstrate that inhibition of expression, or activity of Integrin-Linked Kinase (ILK), a focal adhesion complex protein that also localizes to centrosomes and regulates mitotic spindle assembly (J Cell Biol, 180, 681, 2008) induces centrosome declustering and multi-polar spindle formation, resulting in the death of several breast and prostate cancer cells that harbor multiple centrosomes. Breast and prostatic epithelial cells which do not have multiple centrosomes are resistant to ILK inhibition. ILK regulates the assembly of the Aurora a/TACC-3/chTOG complex, and depletion of centrosomal interactors of ILK, chTOG and TACC-3, also results in centrosome declustering, which is rescued by overexpression of wild-type ILK. Furthermore, although inhibition of actin polymerization with latrunculin also induces centrosome declustering, exposure of cells to both latrunculin and ILK inhibitor, QLT0267, has an additive effect on declustering suggesting a centrosomal role of ILK in mediating centrosome clustering, beyond its focal adhesion role. In conclusion, we have identified a novel cancer cell specific function of ILK in centrosome clustering. Our results suggest that targeting ILK within centrosomes may offer a novel cancer-specific therapeutic strategy.

2158/B535b

Function and Dynamic Reorganization during the Cell Cycle of the Spindle Matrix Protein Chromator in Drosophila.

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The chromodomain protein, Chromator, localizes to interband regions of Drosophila polytene chromosomes at interphase but redistributes during mitosis to form a molecular spindle matrix complex together with three other nuclear derived proteins Skeletor, Megator, and EAST. This complex forms a fusiform spindle structure that persists in the absence of polymerized tubulin and has been proposed based on theoretical considerations of the requirements for force production to help stabilize the microtubule spindle apparatus during mitosis. Previously, we have shown that Chromator regulates chromatin structure and organization of polytene chromosomes at interphase and here we demonstrate the requirement for Chromator function for proper microtubule spindle formation and mitosis in Drosophila larval neuroblasts using two recently generated loss-of-function alleles, Chro71 and Chro612. Our data show that neuroblasts from Chro71/Chro612 brain squash preparations have severe microtubule spindle and chromosome segregation defects that were associated with a developmental small brain phenotype. Time-lapse analysis of mitosis in S2 cells depleted of Chromator by RNAi treatment suggested that the chromosome segregation defects were the results of incomplete alignment of chromosomes at the metaphase plate due to a defective spindle-assembly checkpoint and Mad2 localization, as well as of frayed and unstable microtubule spindles during anaphase. For analysis of the dynamic reorganization of Chromator during the cell cycle we have generated Chromator-GFP and Chromator-mCherry tagged constructs expressed transgenically under native promoter control in order to perform live two-color imaging in syncytial embryos together with fluorescently tagged histone, Ncd, and tubulin. The results obtained show that Chromator redistributes from the chromosomes to the forming spindle matrix as the chromosomes begins to condense in very early prophase and before chromosome alignment at the metaphase plate. The observations are consistent with Chromator playing a role in proper chromosome congression and spindle formation. Supported by NSF grant MCB0817107.
**Kinetochores II (2159 – 2174)**

**2159/B536**

**Aurora B Kinase Phosphorylates Multiple Serine Residues with in the N-Terminus of Hec1 to Regulate Kinetochore-Microtubule Attachment Stability.**  
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Bi-orientation of mitotic chromosomes requires that sister kinetochores capture and stably attach to the plus-ends of spindle microtubules emanating from each of the two spindle poles. Early in mitosis, errors in kinetochore-microtubule attachment are common due to the fact that spindle formation and initial microtubule capture occur simultaneously. Microtubule turnover at kinetochores is high in early mitosis, presumably to induce the release of incorrect kinetochore-microtubule attachments. As cells progress through mitosis, kinetochore-microtubule turnover decreases to allow correctly attached microtubules to generate forces for chromosome movement and for the generation of tension to silence the mitotic spindle assembly checkpoint. Although Aurora B kinase has been implicated in controlling kinetochore-microtubule attachment stability, how the phosphorylation of Aurora B kinetochore targets generates kinetochore-microtubule turnover and promotes attachment error correction remains unclear. We demonstrate that Hec1 of the NDC80 complex is phosphorylated in cells on multiple serine residues, and phosphorylation is dependent on Aurora B kinase. Hec1 phosphorylation at kinetochores is high in early mitosis and is significantly reduced as chromosomes bi-orient, suggesting a role for Hec1 phosphorylation in regulating the attachment stability of kinetochore microtubules. Consistent with this, mutation of these specific serine residues to aspartic acid to mimic constitutive phosphorylation results in destabilized kinetochore-microtubule attachments. Furthermore, western blot analysis of cell lysates treated with specific phosphatase inhibitors suggests that Hec1 phosphorylation is negatively regulated by the Protein Phosphatase-1 family. Our results define a role for Aurora B kinase phosphorylation of Hec1 in microtubule turnover and error correction in early mitosis and a role for the Protein Phosphatase-1 family in dephosphorylation of Hec1 in maintaining stable attachments in late mitosis.

**2160/B537**

**Human Spindly/CCDC99 Couples Kinetochore Dynein Functions to Microtubule Attachment and Checkpoint Signalling.**

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Using expression profiling of synchronised human cells, Whitfield et al. (2002.) and Cho et al. (2001.) have defined genes, whose activity oscillates during the cell division cycle. In order to identify novel regulators of mitosis, we have selected 80 uncharacterised genes and analysed their function by RNAi. By live cell imaging, we have identified 10 genes with defects in progression through mitosis. Here we characterise CCDC99/human Spindly, the mitotic phosphoprotein required for the recruitment of cytoplasmic dynein to kinetochores which is important for microtubule attachment, chromosome movement and mitotic checkpoint signalling. CCDC99 localisation to the outer layer of kinetochores depends on ZW10 and is regulated by microtubule attachment and tension in a dynein- and Aurora kinase B dependent manner, respectively. CCDC99 regulates the turnover of ZW10 at, and removal from, kinetochores but is not required for the removal of the mitotic checkpoint protein MAD2 from bi-oriented chromosomes. Knockdown of CCDC99 causes chromosome congression and spindle formation defects, which trigger a MAD2-dependent mitotic arrest. When ZW10 is depleted by RNAi, either alone or together with CCDC99, however, chromosome congression is less impaired but chromosomes align with wrong attachments. Thus, CCDC99 is not only a kinetochore tether for...
dynein and required for ZW10 removal from kinetochores but is also involved in the regulation of kinetochore microtubule interactions to avoid wrong attachments and potential aneuploidy.

2161/B538
The Clasp Homologue Stu1p Is Required for De Novo Attachment of S. Cerevisiae Chromosomes to Microtubules.
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Accurate chromosome segregation at mitosis depends on bipolar attachment of sister chromatids to spindle microtubules (MTs). The connection between chromosomes and MTs is mediated by kinetochores, which are complex multiprotein structures that assemble on centromeric (CEN) DNA. However, the molecular understanding of how kinetochores bind MTs is still limited. In this paper, we use a combination of high-resolution microscopy and nocodazole-induced kinetochore detachment to study the function of kinetochore MT-associated proteins (kMAPs) in the kinetochore-MT attachment. We find that the yeast CLASP homologue Stu1p accumulates exclusively at detached kinetochores in response to spindle damage and that the Stu1p accumulation at unattached kinetochores does not require intact MTs and activation of the spindle checkpoint. Defective Stu1p does not perturb the kinetochore-MT attachment once this attachment is established, however, fails to accumulate at unattached kinetochores upon nocodazole treatment. Most importantly, interference with the function of Stu1p and Stu2p results in failure in reattachment of kinetochores to spindle MTs. We propose that Stu1p and Stu2p function together in initiating the connection between kinetochores and MTs and thereby promote MT capture at kinetochores. The potential molecular mechanisms are discussed.

2162/B539
The Hec1 Calponin Homology Domain Mediates Kinetochore - Microtubule Attachments In Vivo.
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Kinetochore-MT interactions are critical for chromosome segregation in mitosis. Hec1 is an evolutionarily conserved protein that is one of four members of the Ndc80 complex. Disruption of Ndc80 complex function results in impaired microtubule binding, spindle checkpoint signalling and kinetochore assembly, placing Hec1 at the heart of kinetochore function. In recent years, the N-terminal head domain of Hec1 has emerged as a critical microtubule attachment site in the kinetochore. This domain is comprised of an 80-amino acid positively charged tail and a calponin homology domain, which itself contains conserved basic residues. In vitro data argue that the major binding affinity comes from the unstructured tail; thus it is unclear if the CH domain contributes to microtubule binding at the kinetochore. Employing a knockdown and rescue technique that replaces endogenous Hec1 with rescue plasmids carrying point mutations at charged residues in the CH domain, we find that the CH domain of Hec1 also is important for binding microtubules and aligning chromosomes. Both domains of Hec1 use ionic interactions to bind the microtubule, suggesting that coulombic forces mediate its attachment. We discuss models by which CH domains and unstructured tails work to generate microtubule binding pockets.

2163/B540
KNL-1 Integrates Microtubule Attachment, Checkpoint Signaling, and Recruitment of Protein Phosphatase 1 at the Kinetochore.
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The outer domain of the kinetochore builds an interface where dynamic microtubule interactions are integrated with regulatory pathways, such as the spindle checkpoint, to ensure high fidelity chromosome segregation. The conserved KMN (KNL-1/Mis12 complex/Ndc80 complex) network forms the core microtubule-binding site of the kinetochore and plays a central role in checkpoint signaling. In particular, the KNL-1 subunit of this network is required to target multiple components important for microtubule-binding and checkpoint signaling. To investigate the mechanisms by which KNL-1 acts as an integrative outer kinetochore scaffold, we performed systematic two-hybrid analysis to identify C. elegans kinetochore proteins that interact with KNL-1. In parallel, we developed a single-copy transgene insertion system to selectively express engineered alleles of KNL-1 and assess the consequences on chromosome segregation, kinetochore assembly, mechanical stability of kinetochore-microtubule attachments, and checkpoint signaling. The targeted two-hybrid screen revealed strong interactions between the C-terminal half of KNL-1 and KNL-3 (a subunit of the MIS-12 complex), ROD-1 (a subunit of the RZZ complex) and KBP-5, a non-essential C. elegans KMN subunit of that may be analogous to Zwint in vertebrates. In addition, we discovered that KNL-1 strongly interacts with GSP-1 and GSP-2 (Glc7-type phosphatase), homologs of the Protein Phosphatase 1 catalytic subunit (PP1c). PP1c localizes to kinetochores throughout the eukaryotic kingdom, where it is hypothesized to selectively stabilize correct kinetochore-microtubule attachments by counteracting Aurora B kinase on the adjacent inner-centromeric chromatin. The highly conserved motifs “SILK” and “RRVSF” situated at the N-terminus of KNL-1 are responsible for the binding of GSP-1 and GSP-2 to KNL-1. We are currently investigating the consequences of selectively perturbing the interaction of KNL-1 with PP1c and other outer kinetochore components In Vivo and will report the findings of these efforts.

2164/B541
Molecular Mechanisms of Kinetochore-Microtubule Attachment via the NDC80 Complex.
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The NDC80 complex has been found to be a conserved site of attachment between mitotic chromosomes and microtubules during cell division. Although the subject of intense biochemical, genetic, and structural scrutiny, the molecular mechanisms of this attachment and its ability to couple chromosome motion to microtubule depolymerization remain unresolved. To address this question we have obtained a medium-resolution structure of an engineered NDC80 complex bound to microtubules by cryo-electron microscopy sufficient for accurate molecular docking. Interestingly, we find that NDC80 binds both alpha- and beta-tubulin via an electrostatic mechanism, and that NDC80 molecules also form self-interactions in the context of the microtubule lattice. These data support a biased diffusion, "Hill Sleeve" model of microtubule force-coupling, whereby weak NDC80-microtubule interactions have been optimized to act in coordination to generate robust load-bearing attachments.

2165/B542
Controlled Tubulin Turnover at Kinetochore Microtubule Plus-Ends Is Essential for Chromosome Alignment.
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Accurate chromosome segregation in metazoan cells requires the alignment of sister kinetochores onto the metaphase plate. During chromosome alignment bi-oriented kinetochores
control chromosome movement by regulating the plus-end dynamics of the attached microtubules. However, the factors that control the turnover of tubulin heterodimers at the plus-end of kinetochore-microtubules (kMTs) and the role of these regulatory steps in chromosome alignment are largely unknown. Here we reveal that the human CENP-A NAC/CAD kinetochore complex, and a newly identified kinetochore component Spc34R, are key regulators of kMT plus-end dynamics. Kinetochore-bound Spc34R functions to increase the turnover of microtubule plus-ends. Loss of this activity results in chromosome alignment defects and an increased rate of sister chromatid mis-segregation during anaphase. In contrast, the CENP-A NAC/CAD functions to reduce the turnover of kMT plus-ends. Loss of this activity results in a failure to generate regular chromosome oscillations, which causes disorganization of the metaphase plate. CENP-A NAC/CAD subunits differentially bind to kinetochores in response to changes in kMT dynamics and bind to microtubules in vitro, implying that CENP-A NAC/CAD directly regulates kMT plus-ends. We propose that kinetochores utilize such antagonistic activities to keep kMT plus-end turnover under tight control, and that such control is critical for efficient chromosome alignment.

2166/B543
Centromeric Chromatin Domains Are Defined by an Inverse Relationship to Gene Expression in C. elegans.

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Chromatin assembled using the histone H3 variant CENP-A/CenH3 defines centromeres, the chromosomal domains that direct segregation of the replicated genome during cell division. Both monocentric organisms, with a localized centromere, and holocentric organisms, where centromere activity is distributed along the length of the chromosome, utilize CENP-A chromatin to build kinetochores and direct chromosome segregation. Stable inheritance of CENP-A chromatin domains, with old CENP-A nucleosomes being distributed to daughter strands during DNA replication, and new CENP-A nucleosomes added to replenish the chromatin domain once per division, is postulated to epigenetically propagate centromere identity. Using a controlled mating strategy and quantitative immunoblotting, we show that centromeric chromatin is not inherited through fertilization on sperm chromatin in C. elegans embryos. Instead, CENP-A is loaded de novo onto sperm chromosomes from the oocyte cytoplasm. Using a photobleaching approach, we show that CENP-A chromatin is also not stably inherited through early embryonic cell divisions—all of the CENP-A chromatin present on mitotic chromosomes of a specific division is assembled after anaphase of the prior division. These findings suggest that CENP-A chromatin is specified by a dynamic mechanism in C. elegans. ChIP-Chip based genome-wide location analysis revealed that CENP-A is clustered in regions that are transcriptionally silent in dividing cells but active in post-mitotic tissues. Consistent with this, RNA polymerase II occupancy is strongly inversely correlated with the presence of CENP-A genome-wide. These results show that centromeric chromatin domains are not strictly inherited but are instead defined by the expression landscape of the C. elegans genome. This conclusion sheds light on the evolutionary plasticity of centromere identity in eukaryotes.

2167/B544
Dissecting the Mechanism of Centromere Formation in Xenopus Egg Extracts.

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During cell division, replicated chromosomes segregate equally into daughter cells. Accurate chromosome segregation is essential for proper development and chromosome segregation errors lead to the aneuploid cells that characterize most human cancers. The chromosome segregation apparatus is the microtubule based mitotic spindle. Each mitotic chromosome attaches to the mitotic spindle through a single chromosomal microtubule-binding site called the
kinetochoore. The foundation for the kinetochoore is a region of specialized chromatin called the
centromere. Defects in centromere and kinetochoore function result in improper attachment of
chromosomes to the mitotic spindle and chromosome mis-segregation. The molecular
mechanisms underlying centromere directed kinetochoore assembly is poorly understood. We
have developed a biochemical assay system to reconstitute centromeric chromatin formation In
Vitro using Xenopus egg extracts. Using purified histone proteins and recombinant DNA
substrates we have assembled centromeric nucleosomes and conventional nucleosomes into
chromatin arrays and analyzed their functional properties during centromere assembly. We find
that the reconstituted centromeres efficiently recruit multiple centromere proteins, including
CENP-C, CENP-N, CENP-K, CENP-S, Nsl1, Dsn1 and Nnf1, from the cytosol of Xenopus egg
extracts. The majority of the constitutive centromere proteins recruited from the egg extract bind
to the arrays in a CENP-A dependent manner. However, a subset of the known constitutive
centromere proteins (CENP-S, Nsl1) binds to both CENP-A and histone H3 containing chromatin
arrays. We are currently analyzing centromere and kinetochoore assembly on different chromatin
arrays to characterize the capacities of the arrays for supporting In Vitro centromere and
kinetochoore formation.

2168/B545

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Centromeric chromatin is characterized by the specific incorporation of nucleosomes containing
the histone H3 variant CENP-A. This specialized chromatin domain is responsible for the
assembly of a large constitutive centromere complex, which in turn nucleates the kinetochoore
during mitosis. Although the components of the centromere complex are known in some detail,
little quantitative information is available on the mammalian centromere. This lack of a measure
for size directly impedes our ability to determine the overall architecture of the centromere. A
critical but unknown aspect of centromeric chromatin is the abundance of CENP-A containing
nucleosomes that ultimately supports chromosome tethering to microtubules during mitosis. Here
we use quantitative 3D fluorescence microscopy in living cells to determine cellular CENP-A
distribution. This in conjunction with quantitative immunoblotting against purified CENP-A to
determine whole cell CENP-A concentration provides a direct measure of CENP-A protein copy
number at the centromere. Key to this methodology is the construction of a diploid RPE cell line
that expresses a fluorescently tagged version as the sole source of CENP-A. We have achieved
this by YFP-tagging one of the endogenous CENP-A alleles while deleting the other using a gene
targeting approach. These cells, expressing no untagged CENP-A, display no apparent mitotic
defects and are viable in perpetuity. We will report our results on CENP-A counting experiments
as well as present a novel FACS-based strategy to efficiently and rapidly isolate fluorescent
protein knock-ins based on acquired fluorescence.

2169/B546

Quantitative Analysis of the Propagation of Centromere Identity in G1.
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CENP-A is a variant of histone H3 that incorporates specifically into nucleosomes at centromeres.
CENP-A is required to establish a functional kinetochoore during mitosis, and is thus required for
accurate chromosome segregation. Given that de novo centromeres can form at regions that
contain little or no DNA sequence similarity to canonical centromeric loci, CENP-A has been
proposed to be the epigenetic mark that confers centromere identity. CENP-A is distributed
equally among daughter centromeres during replication, however, no newly synthesized CENP-A
is incorporated at this time. After mitotic exit, CENP-A levels are replenished to pre-replication
levels. Several recent studies have identified proteins involved in CENP-A replenishment. Yet,
little is known about their role in propagating centromeric identity. To characterize the role of
proteins involved in CENP-A replenishment, we decided to quantitatively measure the detailed
spatial and temporal dynamics of CENP-A loading. We expect that depletion of proteins that are directly involved in CENP-A loading reduces both speed and final level of CENP-A recovery. In contrast, depletion of proteins required for propagating centromere identity, its depletion would lead to a complete loss of CENP-A from the centromere, while partial depletion would result in reduced recovery with unperturbed kinetics. We find that CENP-A replenishment takes 6-10 hours, and that it progresses with a rate constant similar to nucleosome incorporation during replication. Depletion of CENP-A inhibits replenishment, but does not affect existing CENP-A, similar to the phenotype we expect to observe for proteins directly involved in CENP-A loading. Interestingly, depletion of HsKNL-2^Mis18BP1 causes complete loss of CENP-A from some centromeres, and partial, but kinetically unperturbed, recovery at others. Furthermore, HsKNL-2^Mis18BP1 disappears from the centromere with the same kinetics as CENP-A appears. Together with the fact that HsKNL-2^Mis18BP1 localizes to centromeres after mitotic exit just prior to other proteins involved in CENP-A loading, this indicates to us that HsKNL-2^Mis18BP1 may act as a temporary epigenetic mark to propagate centromeric identity in early G1.

2170/B547
Construction and Characterization of an Artificial Kinetochore In Vivo.
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Accurate chromosome segregation is ensured by dynamically connecting chromosomes to the plus-ends of spindle microtubules. Key players in this process are kinetochores- large proteinaceous complexes, which assemble on the centromeric DNA and connect the chromosomes to the mitotic spindle. The budding yeast kinetochore consists of more than 80 different proteins, which are further organized in defined subcomplexes and assemble in a hierarchical manner. Despite the small size of the budding yeast centromere which stretches over only 125 bp the temporal and spatial organization of kinetochores is still very complex and poorly understood. To reduce the complexity of the kinetochore we developed a system in which we recruit individual kinetochrome proteins to a yeast mini-chromosome lacking centromeric DNA and ask whether this recruitment is sufficient to promote mini-chromosome segregation in vivo. We show that artificial recruitment of the Dam1 complex to an acentric plasmid can confer mitotic stabilization. This segregation system works independently of the conserved histone H3 variant CENP-A/Cse4p and the inner CBF3 complex and is no longer under the control of the spindle assembly checkpoint. In contrast to that, the mini-chromosomes biorient on the mitotic spindle in an Ipl1/Sli15 dependent manner similar to native chromosomes. Furthermore we demonstrate that this artificial segregation system can also be transferred to native yeast chromosomes. Thus, we have designed a simplified kinetochore In Vivo by directly recruiting force-transducing components to DNA. Future investigations will focus on the purification of these mini-chromosomes from yeast extracts to elucidate the precise protein composition of the artificial kinetochore.

2171/B548
Cell Cycle Control of CENP-A Assembly at Centromeres.
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The centromere is a specialized chromosomal locus responsible for nucleating the kinetochore during mitosis. Centromeric chromatin is characterized by the specific incorporation of CENP-A, a histone H3 variant that is critical for epigenetic inheritance of centromere identity. Using SNAP-tagging we have previously demonstrated that assembly of nascent CENP-A is restricted to a unique cell cycle window in late telophase implicating mitotic events in controlling the maturation of centromeric chromatin. We now show that mitotic passage is dispensable for CENP-A loading. Treatment of cells in G2 phase with cyclin dependent kinase (CDK) inhibitors induces rapid assembly of newly synthesized CENP-A at centromeres. Consistent with canonical loading in G1,
premature CDK inhibition induces recruitment of the CENP-A assembly factors hMis18 and HsKNL-2/M18BP1 to centromeres that precedes arrival of CENP-A. G2 phase loading is dependent on these factors as well as on the CENP-A chaperone HJURP. Critically, drug induced CENP-A assembly does not require proteolysis, uncoupling CENP-A assembly not only from mitotic events but also from the specific involvement of any APC targets that are degraded upon mitotic exit. We hypothesize that the CENP-A assembly machinery is held in check by an inhibitory, CDK dependent mechanism throughout G2 and mitosis and that loss of CDK1 activity in early G1 is directly triggering assembly of nascent CENP-A chromatin.

2172/B549
Identification and Characterization of Factors That Promote Kinetochore Biorientation.
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Accurate chromosome segregation depends on sister kinetochores making bioriented attachments to microtubules from opposite poles. An essential regulator of biorientation is the Ipl1/Aurora B protein kinase that destabilizes improper microtubule-kinetochore attachments. To identify additional biorientation pathways, we previously performed a systematic genetic analysis between the ipl1-321 allele and all non-essential budding yeast genes. One of the mutants was in the MCM21 gene that encodes a kinetochore protein with unknown function. Further characterization revealed a role for Mcm21 in cohesion loading at pericentromeres. In addition, we found that the requirement for Mcm21 in biorientation when Ipl1 function was reduced appeared to be related to its role in pericentromeric cohesion. When pericentromeres were artificially tethered, Mcm21 was no longer needed for biorientation despite decreased Ipl1 activity. Taken together, these data revealed a specific role for pericentromeric linkage in ensuring kinetochore biorientation. We are now trying to identify additional factors that promote pericentromeric cohesion and biorientation using genetic and biochemical approaches. These studies should reveal additional pathways that contribute to these processes as well as further define the link between pericentromeric cohesion and biorientation.

2173/B550
The Spindle Checkpoint Senses Spindle Assymetry.
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The spindle checkpoint is a kinetochore-based surveillance mechanism, which delays anaphase onset in the presence of kinetochore-microtubule attachment defects. The exact nature of the defects that activate the spindle checkpoint is unclear. In particular, it remains controversial whether besides unattached kinetochores insufficient kinetochore tension also engages the checkpoint. Here we tested to which extent the checkpoint also reacts to asymmetric force distribution within the mitotic spindle. To generate “asymmetric” spindles we manipulated the centriole numbers of centrosomes by depleting the human Sas-6 protein, which is required for centriole duplication. We obtained a heterogeneous mix of cells with zero, one or two centrosomes, the latter with distributions of 2:2, 2:1, or 1:1 centrioles. These experiments were carried out in HeLa cells expressing fluorescent markers for centrioles (to count them), microtubules and chromosomes allowing the monitoring of mitotic progression and spindle formation. We find that in cells with one or no centrosomes, anaphase onset and bipolar spindle formation were not abolished, but delayed by several hours, indicating that centrosomes are essential for efficient bipolar spindle formation in human cells. Interestingly, in cells with two centrosomes, the number of centrioles did not affect bipolar spindle formation, but determined the timing of anaphase onset. Indeed, in cells with an asymmetric centriole distribution (cells with 2:1 centrioles but not cells with 2:2 or 1:1 centrioles) we observed a 15 min spindle checkpoint dependent delay in anaphase onset. An asymmetric centriole distribution did not cause attachment defects, as we found no delay in chromosome alignment and no accumulation of
unattached kinetochores. Rather we observed a frequent spindle tumbling, implying an asymmetry in force generation within the mitotic spindle, and kinetochores under reduced tension with lower inter-kinetochore and intra-kinetochore distances. Based on these results we conclude that an asymmetry in centriole numbers at centrosomes leads to force asymmetry within the spindle and that the spindle checkpoint can detect such a force asymmetry in a tension-dependent manner.

2174/B551
Identification of an E3 Ligase That Prevents the Euchromatin Incorporation of the Yeast Cse4/CENP-A Histone H3 Variant.
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High fidelity chromosome segregation during cell division is essential for genomic stability. The centromere (CEN) is the chromosomal locus that assembles the kinetochore (KT), a multi-protein complex required for segregation. A hallmark of all eukaryotic CEN is the presence of nucleosomes containing CenH3, a conserved histone H3 variant that is essential for KT assembly and function. CenH3 is exclusively localized to CEN DNA and the ectopic localization of CenH3 can lead to dicentric chromosomes that can be broken during cell division. However, it is unclear how the exclusive CEN localization of CenH3 is achieved. We previously found that the budding yeast CenH3, Cse4, is degraded by ubiquitin-mediated proteolysis when it localizes to the euchromatin. Here, we identified Cse4 co-purifying proteins and found a putative E3 ubiquitin ligase called Psh1 that interacts with the FACT complex that travels with RNA PolII to disassemble and reassemble nucleosomes during transcription. We show that Psh1 has ubiquitin ligase activity In Vitro and partially mediates Cse4 degradation in vivo. Consistent with a role in degrading mislocalized Cse4, we find that Cse4 mis-incorporates into euchromatin in psh1 mutant cells. Strikingly, Psh1 appears to discriminate between Cse4 and H3 via the Centromere Targeting Domain (CATD) of Cse4 that is not present in H3. Therefore, we propose that Psh1 selectively degrades mis-incorporated Cse4 and thus ensures the exclusive centromeric localization of Cse4.

DNA Replication Defined Cell Cycle (2175 – 2187)

2175/B552
Rac Is Involved in G1/S Transition via Phosphorylation of p27 at Both Thr187 and Ser10 Sites in PI 3-Kinase Pathway but Not in Erk1/2 Pathway.
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In the FGF-2-mediated mitogenic pathway, cell proliferation of corneal endothelial cells (CECs) progresses by removal of p27 from the G1 phase of the cell cycle. The cell cycle-dependent degradation of p27 requires phosphorylation of p27 at Ser10 (major site) and Thr187 (minor site). We had reported that PI 3-kinase is involved in phosphorylation of both sites, thus leading to cell proliferation in response to FGF-2 stimulation. In the present study, we determined whether the ERK1/2 pathway is also involved in the mitogenic pathway via degradation of p27, and we further determined whether Rac is involved in G1/S transition via PI 3-kinase, ERK1/2, or both pathways. When CECs were pretreated with a specific inhibitor for ERK1/2 (U0126) or for Rac (NSC), both inhibitors exerted a marked inhibition of cell proliferation stimulated with FGF-2. Pre-treatment of cells with pathway-specific inhibitors demonstrated that PI 3-kinase and ERK1/2 are parallel pathways during G1/S transition and that Rac is the downstream effector in PI 3-kinase pathways, while Rac is not involved in ERK1/2 pathways as determined by GTP-pulldown assay. Since phosphorylation of p27 at the Ser10 residue is an early event and phosphorylation of p27 at the Thr187 residue is a late event, we used differential kinetics; when cells were stimulated with
FGF-2 for 4, 8 or 12 h, with or without pretreatment of pathway-specific inhibitors, both U0126 and NSC inhibited phosphorylation of p27 at the Ser10 site. The maximum phosphorylation observed in cells treated with FGF-2 for 4 h was greatly reduced by either U0126 or NSC. When late kinetics were tested for phosphorylation of p27 at Thr187, phosphorylation of p27 at the Thr187 site was greatly reduced by either U0126 or NSC. These findings demonstrate that CECs employ two equally dominant pathways (PI 3-kinase and ERK1/2) to remove p27, the major G1 inhibitor in CECs, and that the differential mechanism lies downstream to PI 3-kinase and ERK1/2. Of interest, Rac may play an additional regulatory interface in the PI 3-kinase pathway, which is one major signal transduction in most of cellular activities observed in CECs.

2176/B553
Genomic Studies on DNA Replication, Origins of Replication and Matrix Attachment Regions in Human Chromosomes.
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DNA replication plays an important role in determining the chromatin organization of chromosomes. Genomic studies on the ENCODE pilot area, covering 1% of the human genome, show that time of DNA replication in S phase is an useful parameter for functionally demarcating chromatin domains and boundary elements in chromosomes. The time of replication studies in cancer cells also reveals that as much a 20% of the genome may have interallelic variation in chromatin structure. Extending these studies to identify peaks of origin-centered nascent strands reveals remarkable plasticity in origin usage in human cancer cells. Despite this plasticity, two independent methods of nascent strand purification identify 84 high-confidence origins in the ENCODE pilot area that are used efficiently, increasing the number of well-confirmed human origins of replication suitable for biochemical studies by a factor of ten. These high-confidence origins of replication are usually in early replicating parts of the genome, inside genes and in at rich areas. Chromatin immunoprecipitates reveals that 70% of the high-confidence origins were bound by ORC2 and ORC3, two of the subunits of the human initiator protein complex. However, 30% of non-origin sites are also bound by the same two subunits, indicating that many more chromosomal sites are bound by ORC than are used as origins of replication. Finally, the chromosomal DNA has been postulated to be attached to an insoluble nuclear matrix at specific matrix-attachment regions (MARs) that act as boundary elements between chromosomal domains. Genomic approaches show that MARs are associated with transcriptionally active genes, with origins of replication and with boundary elements between chromatin domains. In summary, these genomic studies reveal significant plasticity of chromosome structure superimposed on more fixed landmarks like high-confidence origins of replication and subsets of MARs.

2177/B554
Dbf4/Cdc7 Kinase Is a Target of ATM and ATR in the S-Phase Checkpoint.
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Genome instability is a hallmark of the malignant phenotype and a driving force for tumorigenesis. The S-phase checkpoint is a principle defense mechanism to keep genome stability. The detailed mechanism by which the S-phase checkpoint is operated in mammalian cells remains unclear. Dbf4/Cdc7 is a protein kinase complex that is required for DNA replication (S-phase) initiation. Our preliminary data suggest an important role of Dbf4/Cdc7 in the S-phase checkpoint. We found that DNA damages (IR, HU, UV) induce hyper-phosphorylation of human Dbf4. Phosphorylated Dbf4 and Cdc7 remain a stable complex and accumulate in a chromatin-enriched fraction response to replication stress. Furthermore, hyper-phosphorylation of Dbf4 is dependent on ATM and ATR. We also demonstrated that Dbf4 is phosphorylated by ATM and ATR in vitro.
In conclusion, we showed a promising mechanism by which the S-phase checkpoint is operated through regulating the Dbf4/Cdc7 pathway in mammalian cells. These studies will clarify molecular pathways that link damage signal transduction through Dbf4/Cdc7 to the effector proteins to regulate S-phase checkpoint. They will help to elucidate the overall mechanism of the S-phase checkpoint in mammalian cells and shed light on the cellular mechanisms that control genome stability and prevent cancer.

2178/B555

Tissue Specific Regulation of Drosophila Cdt1 By Cullin E3 Ubiquitin Ligases.

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To maintain genome integrity during cell cycle progression, each individual origin of replication is carefully regulated to initiate DNA synthesis only once during a given S phase. This regulation is achieved through the assembly and disassembly of the pre-replicative complex (pre-RC). Pre-RC assembly involves recruitment of the MCM DNA helicase to origins of replication, and pre-RC disassembly at the onset of S phase prevents reloading of the helicase and subsequent re-replication. Cdt1 is an important member of the pre-RC that is necessary for MCM recruitment, and Cdt1 misregulation has been linked to over-replication in many organisms. In addition, Cdt1 and its inhibitor Geminin are over-expressed in various cancers, consistent with the idea that genome instability due to pre-RC misregulation can contribute to cancerous growth. Cdt1 is also regulated by ubiquitin-mediated proteolysis, being a substrate of both Cul1- and Cul4-dependent E3 ubiquitin ligases in mammalian cells. To better understand the significance of the different modes of Cdt1 regulation during development, we engineered transgenic Drosophila expressing mutant versions of Cdt1 that cannot interact with Cul1 and/or Cul4. I found that the Cul4-binding mutant of Cdt1 (ΔPIPCdt1) is partially stabilized during S phase and causes defects in tissue morphology, indicating that Cul4-dependent degradation of Cdt1 is crucial for normal development. ΔPIPCdt1 was able to rescue the lack of DNA replication in Cdt1 null mutant embryonic cells, but did not support progression of these cells into mitosis. This indicates that Cul4 regulation of Cdt1 is needed for normal S phase progression in proliferating diploid cells. In contrast, the expression of ΔPIPCdt1 did not affect endocycle progression in ovary follicle cells. Endocycles consists only of S and G phases without cell division and result in polyploid cells. We are currently exploring whether other modes of Cdt1 regulation, such as Geminin binding, act redundantly with Cul4-mediated destruction of Cdt1 during endocycles. Finally, a ΔPIPCdt1 variant that also cannot interact with Cul1 is only partially stabilized during S phase, suggesting the possibility of another regulator of Cdt1 degradation.

2179/B556

Recombination and Slowing of Replication Forks.

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The S phase DNA damage checkpoint slows the rate of replication fork progression in response to DNA damage. In fission yeast, replication slowing relies on Rad3, the central checkpoint kinase and homolog of the metazoan ATR kinase, and Cds1, the S-phase specific transducer kinase. However, players downstream of these kinases and mechanism utilized to slow replication forks is not well understood. We wished to identify proteins required for slowing and determine how replication fork slowing is accomplished in fission yeast. We genetically identified 16 genes involved in slowing which act downstream of Cds1; many of these genes are implicated in replication fork stability and the negative regulation of recombination. They include the structure-specific endonuclease Mus81 and the RecQ helicase Rqh1. Interestingly, abrogation of recombination by removing Rhp51, the Rad51 recombinase homolog, suppresses the slowing defect displayed by the rqh1 mutant, but not that of the mus81 mutant. These results define an epistatic pathway in which mus81 is epistatic to rhp51 which in turn is epistatic to rqh1. Our data suggests sister-chromatid exchange plays an important role in replication of damaged DNA. We
propose that fission yeast reduce replication fork rate at sites of DNA damage by a mechanism that involves inhibition of recombination and thus prevention of sister-chromatid exchange mediated bypass of DNA damage.

2180/B557

Spindle Pole Body Dynamics at Medial Nuclear Division Arrest.
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In S. cerevisiae, the cell cycle can be interrupted after DNA replication at “medial nuclear division arrest” (MND). MND arrest can result from DNA damage (e.g. in cdc13-1 at 30°C) and from inhibition of the anaphase promoting complex (e.g. upon depletion of Cdc20p). at MND, cells have large buds and the DNA mass often extends across the bud neck. Time-lapse imaging of DAPI-stained cells at MND has shown that the chromatin mass transits between the mother and daughter for hours (Palmer et al., JCB 109, 3355 (1989)). To understand the role of the mitotic spindle, we have arrested cells at MND and have used time-lapse imaging to localize FP-tagged tubulin and chromatin, as well as tagged proteins of the spindle pole body (SPB) and nuclear pore complex. We observe that the nuclear envelope extends into both the mother and bud. within this envelope, the spindle has extensive rotational freedom, yet retains a near-constant length and transects the chromatin mass, with both ends terminating at SPBs in the nuclear envelope. As chromatin transits occur, the nuclear envelope continues to extend into both mother and bud, but the orientation of the spindle and the position of the SPBs shift abruptly, with the most dramatic shifts causing one or both SPBs to traverse the bud neck. There is no suggestion that the SPBs detach from the nuclear envelope. We therefore conclude that the SPBs can be highly mobile within the plane of the nuclear envelope.

2181/B558

The Elg1-RFC Complex Functions in Sister Chromatid Cohesion Separately from Ctf18.
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Ctf7/Eco1 is an essential yeast factor that is required to pair sister chromatids together during S phase. Cohesion establishment occurs via Ctf7/Eco1 acetylation of Smc3 - a core cohesin subunit. Of interest is that cells also exhibit an anti-establishment activity in that Pds5 and Rad61 mutation can bypass the essential role of Ctf7/Eco1 acetyltransferase activity. Here, we identify several DNA replication factors as either establishment or anti-establishment factors. Replication factor C (RFC) complexes catalyze the loading of PCNA-like sliding clamp complexes onto primed DNA. RFC complexes consist of four small subunits plus one of the four interchangeable large subunits. Rfc1-RFC is the only essential RFC complex and is utilized for processive DNA replication. Ctf18-RFC, Elg1-RFC and Rad24-RFC function in various DNA repair processes with many overlapping cellular duties. Of the four large RFC subunits, prior studies revealed that Ctf18 promotes sister chromatid pairing. We tested and found that Elg1 does not compensate for loss of Ctf18 function in cohesion- but rather exhibits the opposite function such that deletion of ELG1 rescues ctf7 mutant cell cohesion establishment phenotypes. Thus, Elg1 is a new anti-establishment factor -linking this activity to the DNA replication fork. Here, we demonstrate a further role for RFC complexes in cohesion in that mutation of rfc5 also rescues ctf7 mutant phenotypes. The role of Elg1 and Rfc5 are specific to anti-establishment in that rfc5 mutant gene in combination with cohesion structural (SMC3, MCD1, PDS5) or deposition mutant (SCC2) exacerbates the slow growth phenotypes associated with these cohesion mutants. This evidence suggests that small RFC subunits interact with Elg1 for anti-establishment processes and that Ctf18 functions by itself or in a separate complex to promote cohesion establishment.

2182/B559

AAA-ATPase Cdc48 and Cofactors Npl4-Ufd1 Facilitate G1 Progression in Response to Heat Stress.
Cdc48 is a member of the AAA (ATPase associated with a variety of activities) ATPasesuperfamily and is known to be involved in many processes, including ER-associated protein degradation, membrane fusion, and mitosis. We examined the phenotypes of temperature-sensitive *cdc48-3* mutant in the budding yeast *Saccharomyces cerevisiae* and found that the mutant is largely arrested at mitosis at 37°C. However, the mutant is also perturbed in G1 progression at 38°C, with delayed budding and defect in actin polarization. Interestingly, expression of the G1 cyclin Cln1, but not Cln2 and Cln3, is delayed in *cdc48-3* at 38°C. Reporter assays show that the activity of *CLN1*, but not *CLN2*, promoter is reduced in *cdc48-3* at 38°C. By examining the known adaptors for Cdc48, we found that temperature-sensitive *npl4-1* and *ufd1-2* mutants also exhibit G1 phenotypes similar to *cdc48-3* at 38°C, but not 37°C. The promoter activity of *CLN1* is also reduced in *npl4-1* and *ufd1-2* at 38°C, indicating that Npl4-Ufd1 complex mediates the function of Cdc48 in G1. In addition, overexpression of a dominant-negative Cdc48 also delays G1 progression at 38°C, but not 30°C, indicating that the G1 phenotypes is related to heat stress. Our results demonstrate that Cdc48 promotes the expression of *CLN1* during recovery from the transient G1 arrest in response to heat stress and that this function is mediated by Npl4-Ufd1 adaptor complex.

**2183/B560**

**A Novel Outcome for Signalling through the MAPK Pathway.**

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The primary endpoint for signalling through the canonical Ras-Raf-MEK-MAP kinase cascade is ERK activation. Here we report an alternative outcome for signalling through this pathway which is independent of the pathways’ recognised downstream effector ERK but relies specifically on a truncated isoform of MEK1. Activation of the MAP kinase pathway during G2 phase by growth factors (epidermal growth factor - EGF) or phorbol esters (12-O-Tetradecanoylphorbol 13-acetate - TPA) results in delayed G2/M phase progression. Previously we have identified a novel proteolytically cleaved form of MEK1 (tMEK - truncated MEK1), which has lost its N-terminal ERK binding domain. We show that induction of tMEK during G2 phase delays entry into mitosis. RNAi mediated knockdown in somatic cells or knockout in MEFs of MEK1 but not MEK2 results in loss of the G2 delay. The G2 delay can be recapitulated with addition of recombinant tMEK protein. Investigation of G2 checkpoint proteins demonstrated a lack of involvement of classical G2 phase DNA damage/stress response molecules such as p38MAPK, chk1/2 and ATM/ATR kinases. Exploring further downstream however, the cdc25 group of dual specificity phosphatases demonstrated rapid degradation specifically of cdc25B in response to TPA or EGF treatment. This degradation could be blocked by the addition of the protease subunit inhibitor MG132 or interestingly, the addition of the MEK1/2 inhibitor U0126. Addition of recombinant tMEK protein also resulted in reduced stability of the cdc25B isoform suggesting that tMEK may mediate its effect of delaying G2/M progression through the destabilisation of the key cell cycle regulator cdc25B. The existence of a novel form of MEK1 with functions that are independent of its normal effector ERK, is of great biological significance. In many cancers, signalling through the MAPK pathway involving Ras-Raf-MEK-ERK is enhanced, usually via oncogenic mutations in Ras or Raf, providing proliferative and pro-survival advantages to cells. The existence of another function for components of this pathway unrelated to ERK activation provides an entirely novel spectrum of possible outcomes for aberrant activation of this pathway.

**2184/B561**

**Chk1 Phosphorylation By Cdk1 Is Required for the Adequate Activation of Cdk1.**
Chk1, one of critical transducers in DNA damage/replication checkpoints, prevents entry into mitosis through the inhibition of Cdk1 activity. However, it remained unclear how this inhibition is cancelled at the G2/M transition. Recently, we reported that Chk1 is phosphorylated at Ser286 and Ser301 by Cdk1 during mitosis. Here, we show that mitotic Chk1 phosphorylation is accompanied by Chk1 translocation from the nucleus to the cytoplasm in prophase. Exogenous Chk1 mutated at Ser286 and Ser301 to Ala (S286A/S301A) was mainly observed in the nucleus of prophase cells although such nuclear accumulation was hardly observed in the Chk1 wild type (WT) case. Induction of S286A/S301A resulted in dramatic delay of mitotic entry, compared with WT. Biochemical analyses using immunoprecipitated Cyclin B1/Cdk1 complexes revealed S286A/S301A expression to block the adequate activation of Cdk1. In support of this, S286A/S301A expression retained Wee1 at higher levels and Cdk1-induced phosphorylation of Cyclin B1 (at Ser126) and vimentin (at Ser55) at lower levels. A kinase dead-version of S286A/S301A also localized predominantly in the nucleus but lost the ability to delay mitotic entry. These results indicated that Chk1 phosphorylation by Cdk1 participates in cytoplasmic sequestration of Chk1 activity, which releases the Cdk1 inhibition in the nucleus for maintaining the proper mitotic entry.

2185/B562

A Novel Human Centrosomal Protein, AIBp Interacts with the Kinase Domain of Aurora-A and Functions as a Positive Regulator.

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Tremendous progresses have been made towards understanding the regulation and function of Aurora-A involved in chromosome alignment, centrosome maturation, mitotic spindle assembly and regards to an oncogene. Aurora-A is also known to bind to several other proteins which affect either up-regulation or down-regulation of Aurora-A, implicated in its activation/inactivation and localization. However, how these different binding signals are integrated to regulate Aurora-A is not properly known. In this report, using pGBT9-Aurora-A as bait in a low-copy yeast two-hybrid system, we identified a novel human centrosomal protein AIBp (Aurora-A Binding Protein). The full-length AIBp cDNA contains a 1117-bp open reading frame that encodes a 371-amino acid polypeptide. Using semi-quantitative PCR analysis, low-level expression of AIBp was detected in normal human tissues. Our data showed that the C-terminus of AIBp can directly interact with the C-terminus of Aurora-A, and kinase activity was required for this interaction. We also demonstrated that AIBp specifically interact with Aurora-A, but not Aurora-B and Aurora-C. In addition, the co-immunoprecipitation and GST-pull down were performed to confirm the specific interaction regions between AIBp and Aurora-A. Further, immunofluorescence assay revealed that AIBp and Aurora-A partially co-localize at G2 and prophase. In Vitro kinase assay demonstrated that AIBp was phosphorylated by Aurora-A. Functional assay also demonstrated the binding of AIBp to Aurora-A promoted enhanced its kinase activity. Upon siRNA-mediated elimination of AIBp from HeLa cells, results in less concentrated and mislocated the Aurora-A to spindle pole and spindle. Furthermore, we demonstrated that both AIBp and Aurora-A are co-overexpression in various brain tumors. All together, these studies demonstrate that AIBp may not only be required for initial activation and dynamic movement of Aurora-A at centrosomes and spindle apparatus during cell cycle but also be important in brain tumorigenesis.

2186/B563

A Decrease in Cyclin B1 Levels Leads to Over-Replication in DNA-Damage Induced Senescence.
Polyploidy in cancer cells are involved in tumorigenesis via leading to aneuploidy. Although abrogation of cell division accompanying over-replication of DNA is thought to result in polyploidization, the mechanisms underlying induction of polyploidy have been hitherto largely unclear. In this study, to investigate a pathway for DNA damage-induced polyploidization, we analyzed the regulation for inhibition of mitotic entry upon treatment with DNA-damaging anticancer drugs. Treatment with bleomycin induced over-replication at low cytotoxic doses. In the early phase of treatment with bleomycin, mitotic entry was inhibited through tyrosine phosphorylation of CDK1 along the ATM/ATR pathway. The inhibitors of the ATM/ATR pathway, caffeine and debromohymenialdisine, inhibited bleomycin-induced over-replication through abrogation of bleomycin-induced G2 arrest and promoted cell death instead of over-replication. These results suggest that the ATM/ATR pathway acts as a molecular switch for regulating cell fates, flipping between cell death via progress into mitosis, and over-replication via sustained G2 arrest upon DNA damage. Despite dephosphorylation of CDK1, mitosis was still inhibited. Notably, the level of cyclin B1 was decreased in the late phase of treatment. A clone to express a live cell marker of endogenous cyclin B1 was generated, and time-lapse imaging of the clone cells revealed that cyclin B1 was degraded in G2-arrested cells upon bleomycin treatment. Furthermore, a subcytotoxic concentration of Adriamycin gave rise to over-replicated cells having the features of senescence, such as enlarged and flattened cell shape and activated β-galactosidase activity. During the over-replication induced by Adriamycin, cyclin B1 levels were decreased. These results suggest that decreased levels in cyclin B1 trigger over-replication in Adriamycin-induced senescent cells. In conclusion, these results suggest that a decrease in cyclin B1 levels is induced by DNA damage, resulting in over-replication in DNA damage-induced senescence.

2187/B564
Dimerization of CENP-J Governs Centrosome Cohesion Plasticity.
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Coupling with cell cycle, centrosome cohesion and segregation are accurately regulated to prevent an aberrant separation of duplicated centrosomes and ensure the correct formation of bipolar spindles. CENP-J is a centrosome protein with five coiled-coil domains and plays an important role in control of brain size in autosomal recessive primary microcephaly. Previous studies showed that CENP-J interacts with tubulin and controls centriole length. Here we reported that CPAP forms dimmer in interphase and the fifth coiled-coil domain of CENP-J is required for its dimerization. Moreover, this self-interaction is required for maintaining centrosome cohesion and preventing the centrosome from splitting before G2/M phase. In addition, both monomeric and polymeric CENP-J were required for normal mitosis, suggesting that the self-interaction of CENP-J is regulated during the cell cycle. Significantly, our results provide evidence that CENP-J is phosphorylated during mitosis and this phosphorylation releases its inter-molecular interaction. Taken together, these results suggest that cell cycle regulated phosphorylation orchestrates dynamics of CENP-J molecular interaction and centrosome splitting to ensure the genomic stability in cell division.

Golgi Complex (2188 – 2200)

2188/B565
ArfGAP1 Generates an Arf1 Gradient on Continuous Lipid Membranes Displaying Flat and Curved Regions.
ArfGAP1, which promotes GTP-hydrolysis in the small G protein Arf1 on Golgi membranes, interacts preferentially with positively curved membranes through its ALPS motifs. This should influence the spatial distribution of Arf1-GTP when flat and curved regions coexist on a continuous membrane, notably during COPI vesicle budding. To test this, we pulled tubes from giant vesicles using molecular motors or optical tweezers. Arf1-GTP distributes on the vesicles and on the tubes, whereas ArfGAP1 was found exclusively on the tubes. Decreasing the tube radius revealed a threshold of $R = 35$ nm for the binding of the ALPS motifs of ArfGAP1. Mixing ArfGAP1 with Arf1-GTP results in smooth Arf1 gradients along tubes of tens of micrometers in length. Because the size of COPI buds is much smaller, Arf1-GTP diffusion could readily compensate for the localized loss of Arf1 during budding and contribute to the stability of the coat until fission.

2189/B566
Phospholipase Complex Pafah IB Regulates the Structure and Function of the Golgi Complex.
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Phospholipase A$_2$ (PLA$_2$) activity has been reported to maintain the organization and proper function of the mammalian Golgi complex by the regulation of membrane tubule formation. We have identified platelet activating factor acetylhydrolase (PAFAH) Ib ($\alpha_1$, $\alpha_2$, LIS1/$\beta$) as a PLA$_2$ complex that stimulates membrane tubules, modulates Golgi structure, and regulates trafficking from the Golgi complex. Purified $\alpha_1$ and $\alpha_2$ subunits, but not catalytically inactive mutant counterparts, were capable of inducing Golgi membrane tubule formation in an In Vitro reconstitution system. Both $\alpha_1$ and $\alpha_2$ partially localized on Golgi membranes in vivo, and over-expression of these subunits disrupted Golgi morphology. Over-expression of $\alpha_1$ or $\alpha_2$ catalytically inactive and/or mutants unable to bind LIS1/$\beta$ demonstrated that both catalytic activity and LIS1/$\beta$ binding, a potential link between membrane curvature and dynein regulation, are important for the organization of the Golgi complex. siRNA-mediated knockdown of $\alpha_1$ and $\alpha_2$ fragmented the Golgi ribbon into mini-stacks and inhibited the reassembly of an intact Golgi ribbon after recovery from Brefeldin A. Additionally, knockdown of $\alpha_1$ and $\alpha_2$ significantly inhibited trafficking of soluble and transmembrane cargo from the trans Golgi network (TGN) to the cell surface. These results demonstrate that PAFAH Ib is involved in generating membrane tubules and regulating the functional organization of the Golgi complex. Furthermore, these results support the hypothesis that membrane tubule formation and trafficking is achieved by changes in phospholipid composition.

2190/B567
ARL1 in Saccharomyces cerevisiae: Exploration of the Grip Domain Binding Site.
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Ar1, a guanine-nucleotide binding protein, regulates both ion homeostasis and membrane traffic in the yeast, Saccharomyces cerevisiae. In addition, Ar1 in various species, from fungi, plants, and mammals, has been shown to interact with golgin proteins via the GRIP domain, a conserved region of about 50 amino acid residues. In efforts to determine whether these two functions of yeast Ar1 require interaction with the sole GRIP domain-containing protein in yeast, Imh1, we made mutations similar to those shown to disrupt interaction of Ar1 with a GRIP domain partner in other organisms. Specifically, we generated three mutants, $ARL1[552G]$, $ARL1[81A]$, and $ARL1[82G]$. A yeast strain deleted for chromosomal $ARL1$ was transformed with a plasmid containing one of the three new mutants. The same strain was also transformed with empty
vector or wild type ARL1 as negative and positive controls, respectively. The transformants were then tested for their ability to grow in the presence of hygromycin B (Hyg B), a measure of Arl1’s role in ion homeostasis; and for their ability to suppress the aberrant secretion of carboxypeptidase Y (CPY), a measure of Arl1’s role in membrane traffic. Mutation of the F52 and Y82 residues adversely affected the ability of Arl1 to suppress both Hyg B sensitivity and CPY secretion; conversely, mutation of the C81 residue resulted in a protein that behaved like wild type Arl1 in both assays. Mapping of these residues onto the crystal structure of yeast Arl1 bound to GDP revealed that both F52 and Y82 are part of a hydrophobic patch on the surface whereas C81 points away from this region. We conclude that both aspects of Arl1 function, ion homeostasis and membrane traffic, require interaction with Imh1. This work was supported by a grant from the National Science Foundation (MCB-0641266).

2191/B568
Bile Acids Modulate Golgi Structure in Gastrointestinal Cancer via a PKC\(\eta\) /PKD-Dependent Pathway,
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Background Deoxycholic acid (DCA) is a secondary bile acid which modulates signaling pathways in epithelial cells. DCA has been implicated in pathogenesis of colon carcinoma, particularly by activation of the protein kinase C (PKC) pathway. Ursodeoxycholic acid (UDCA) a tertiary bile acid has been observed to have chemopreventative effects. Objective The aim of this study was to investigate the effect of DCA and UDCA on the subcellular localisation and activity of Protein Kinase C\(\eta\) (PKC\(\eta\)) and its downstream effects on Golgi structure in a colon cancer cell model. Methods Subcellular localisation and activity of PKC\(\eta\) was assessed by immunofluorescence and Western Blot analysis respectively. Golgi were visualised by immunofluorescence and Golgi fragmentation was quantified using High Content Analysis. The involvement of the glucocorticoid receptor (GR) was assessed using pharmacological inhibition and an siRNA approach. Tissue microarrays were utilised to assess Golgi fragmentation in normal, ulcerative colitis (UC) and colon cancer tissue. Results PKC\(\eta\) expression was localised to the Golgi in HCT116 colon cancer cells. DCA induced fragmentation of the Golgi in these cells by activation of PKC\(\eta\) and its downstream effector PKD. Pre-treatment of cells with UDCA or a glucocorticoid, dexamethasone, inhibited DCA-induced PKC\(\eta\)/PKD activation and Golgi fragmentation. Knockdown of glucocorticoid receptor (GR) expression using siRNA or inhibition using the GR antagonist mifepristone, attenuated the inhibitory effect of UDCA on Golgi fragmentation. Elevated serum and faecal levels of DCA have been previously reported in patients with ulcerative colitis (UC) and colon cancer. Analysis of Golgi architecture In Vivo using tissue microarrays revealed Golgi fragmentation in UC and colorectal cancer tissue. Conclusion In conclusion we have demonstrated that DCA can disrupt the structure of the Golgi, an organelle critical for normal cell function. Inhibition of this DCA-induced Golgi fragmentation by UDCA was mediated via the glucocorticoid receptor. This represents a potential mechanism of observed chemopreventative effects of UDCA in benign and malignant disease of the colon.

2192/B569
The COG Complex, Rab6 and COPI Define a Novel Golgi Retrograde Trafficking Pathway That Is Exploited By SubAB Toxin.
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Toxin trafficking studies provide valuable information about endogenous pathways of intracellular transport. Subtilase cytotoxin (SubAB) is transported in a retrograde fashion through the endosome to the Golgi and then to the ER, where it specifically cleaves the ER chaperone
BiP/GRP78. To identify the SubAB Golgi trafficking route, we have used siRNA-mediated silencing and immunofluorescence microscopy in HeLa and Vero cells. Knockdown of subunits of the Conserved Oligomeric Golgi (COG) complex significantly delays SubAB cytotoxicity and blocks SubAB trafficking to the cis-Golgi. Depletion of Rab6 and β-COP proteins causes similar delay in SubAB-mediated GRP78 cleavage and did not augment the trafficking block observed in COG KD cells, indicating that all three Golgi factors operate on the same “fast” retrograde trafficking pathway. SubAB trafficking is completely blocked in cells deficient in the Golgi SNARE Syntaxin5 and does not require the activity of endosomal sorting nexins SNX1 and SNX2. Surprisingly, depletion of Golgi tethers p115 and golgin-84 which regulates two previously described COPI vesicle-mediated pathways did not interfere with SubAB trafficking, indicating that SubAB is exploiting a novel COG/Rab6/COPI-dependent retrograde trafficking pathway.

2193/B570

Trafficking of Golgi-Resident Enzymes Studied By Rapamycin-Induced Protein Dimerization and Photoactivable Golgi Enzymes.

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The Golgi apparatus is the hub of the secretory pathway and maintains its organization amidst the constant flux of secretory cargo. In steady state, Golgi enzymes are enriched in the Golgi complex and thus were traditionally viewed as stably anchored in the Golgi. However, in recent years, several lines of evidence suggest that the Golgi apparatus is a highly dynamic structure with the putative Golgi-resident enzymes continuously cycling both within the Golgi apparatus and also between the Golgi and the endoplasmic reticulum (ER). We used a combination of rapamycin-induced protein-dimerization and photoactivable fluorescent protein (PACHerry) tagged-Golgi enzymes to study the trafficking itinerary of Golgi enzymes and the relationship of Golgi to the ER. FKBP (FK506-Binding protein) forms a tight complex with FRB (FKBP-rapamycin-associated protein) in presence of rapamycin. A salient prediction of constitutive recycling of Golgi-enzymes is that in presence of rapamycin, FRB-tagged Golgi enzymes visiting the ER will get trapped in ER by an ER-retained protein fused to FKBP (Invariant chain-FKBP-Cerulean). The Golgi-resident pool of Mannosidase II-FRB-PACHerry (ManII-FRB-PACHerry) was first selectively photoactivated, all fluorescent molecules outside the Golgi was photobleached using iFRAP and the spatial and temporal dynamics of the Golgi-pool of ManII-FRB-PACHerry was monitored. The Golgi localized ManII-FRB-PACHerry completely relocated to the ER over a time period of 90-120 minutes. We see similar relocalization of Golgi enzymes to ER when we use ManII-FRB-Venus and other FRB-tagged Golgi enzymes. Inhibiting retrograde trafficking using a PLA2-inhibitor prevented the redistribution of Golgi enzymes. The presence of an ER-trap also prevented the appearance of scattered Golgi fragments following microtubule disruption by nocadazole; instead the ManII-FRB-PACHerry/Venus redistributed to the ER, indicating that nocadazole induced fragmentation of Golgi involves accumulation of the constitutive recycling Golgi enzymes at the ER exit-sites. Our results show that Golgi enzymes constitutively recycle under physiological conditions and the maintenance of Golgi depends on its continuous biogenesis from the ER.

2194/B571

Rab6 Regulates Golgi Vesicle Release and Cisternal Homeostasis.

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Taking an epistatic approach to characterize critical functions of Golgi associated Rab proteins in mammalian cells, we have shown previously that Rab6 is required for the cytoplasmic accumulation of Golgi-derived vesicles in response to depletion of the putative retrograde tether, COG complex (Sun et al., MBoC, 2007). By electron microscopy, we now report that Rab6-depletion produced a striking accumulation of arrested, budding structures that remain continuous with Golgi membranes. These structures were coated and found in association with both
elements of the trans Golgi network (TGN) and cisternal elements of the Golgi stack. Morphologically, clathrin was identified as the TGN associated coat and COPI as the cisternal coat. In addition, in thin sections, accumulation of “free” Golgi associated coated vesicles were apparent. Tomographic characterization of 300 nm thick sections indicated that many of these are free vesicles devoid of any membrane continuity with the Golgi apparatus. Few, if any, Golgi associated vesicles were observed in control HeLa cells and continuities between Golgi cisternae were not observed in either control or Rab6 depleted cells. We suggest that Rab6 regulates vesicle scission and separation from the Golgi apparatus. Importantly, these observations suggest that vesicle formation is a normal Golgi physiological process that is likely obscured by rapid vesicle consumption. Furthermore, in survey thin sections, we found a significant increase in the number of cisternae per Golgi stack: 4.2 ± 0.32 cisternae per stack in siControl cells versus 6.8 ± 0.46 cisternae per stack in siRab6 cells. We suggest that the change in cisternal homeostasis is the outcome of rebalanced membrane trafficking through the organelle. Based on our electron microscopy, Rab6 depletion should have major effects on membrane trafficking/recycling mediated by intra-Golgi vesicles. Contrary to this expectation, we found by confocal fluorescence microscopy that the cis to trans polarity of Golgi proteins was maintained. In conclusion, we suggest that cisternal maturation/progression recalibrated to a new set point can most readily explain these results.

2195/B572
Use of 3D Imaging to Establish the Dynamics of Coats Containing Clathrin and Either AP-1 or AP-3 Adaptors in Living Cells.
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We generated an automated tracking algorithm to determine the three dimensional trajectories of fluorescent objects within live cells imaged using fast 3D spinning disk confocal fluorescence microscopy. With this tracking scheme we can locate an organelle labeled with ~20 or more fluorescent proteins with a precision of less than 100 nm along each of the three dimensions within a period of less than 0.5 seconds. Location along the Z axis was established using single Gaussian curve fitting of the local fluorescence intensities determined on 5 or more sequential focal planes spaced ~350 nm. Using this tool, we determined the temporal and spatial behavior of AP-1 and AP-3 containing clathrin structures in BSC1 monkey cells expressing σ1-EGFP or σ3-EGFP together with LCa-tomato, respectively. We found that either type of clathrin adaptors have strong colocalization with clathrin and the adaptor structures that appear and disappear within our imaging window have average lifetimes of ~30 seconds. We also found that in some cases multiple AP-1 or AP-3 clathrin containing coats displaying independent dynamic behaviors are simultaneously located on the same membrane-bound organelle. Finally, intracellular movement of these organelles is mainly performed by microtubule dependent molecular motors.

2196/B573
Actomyosin Stretches the Golgi By Attachment to a Protein That Binds Phosphatidylinositol-4-Phosphate.
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Golgi membranes, from yeast to humans, are uniquely enriched in phosphatidylinositol-4-phosphate (PtdIns(4)P), although the role of this lipid remains poorly understood. Using a proteomic lipid binding screen, we identify a novel PtdIns(4)P-binding protein that depends upon PtdIns(4)P for its Golgi localization. We further show that this protein binds the unconventional
myosin MYO18A, thus connecting the Golgi to F-actin. We demonstrate that this linkage is necessary for normal Golgi trafficking and morphology. The evidence suggests that this protein binds to PtdIns(4)P-rich trans-Golgi membranes and MYO18A, conveying a tensile force required for efficient tubule and vesicle formation. Consequently, this tensile force stretches the Golgi into the extended ribbon observed by fluorescence microscopy and the familiar flattened form observed by electron microscopy.

2197/B574

An Expansive Scy: Scyl1 Regulates Golgi Morphology.
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Membrane trafficking is a defining feature of eukaryotic cells, and is essential for the maintenance of organelle homeostasis and identity. We previously identified Scyl1, a member of the Scy1-like family of catalytically inactive protein kinases as a high-affinity binding partner of coatamer I (COPI) coats, which control Golgi to endoplasmic reticulum trafficking and Golgi homeostasis (Burman et al., J. Biol. Chem., 2008). We now demonstrate that Scyl1 regulates Golgi morphology and that loss of Scyl1 function leads to a Golgi expansion phenotype. Both the surface area and volume of the Golgi is increased in Scyl1-depleted cells, but the continuity and polarity of the Golgi is unperturbed. At the ultrastructural level we observe an increase in Golgi length and cisternal luminal width, while the number of Golgi cisternae remain unchanged. Of interest, we have also found that Scyl1 possess many of the characteristics assigned to the golgin family of proteins. Golgins form a detergent resistant protein network that controls Golgi homeostasis and disruption of this network by knock down of the golgin p115 disrupts the Golgi localization of Scyl1. These data demonstrate that Scyl1 is a regulator of Golgi homeostasis. Given that loss of function mutations in Scyl1 result in the mouse neurodegenerative disease model mdx, our data suggest that dysregulation of Golgi morphology could contribute to the disease process.

2198/B575

Caspase-2 Cleavage of Cert during Apoptotic Stress Modulates Sphingomyelin Synthesis.
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The mammalian Golgi apparatus is composed of multiple stacks of cisternal membranes organized laterally into a ribbon-like structure, with close apposition of trans Golgi regions with specialized endoplasmic reticulum (ER) membranes. These contacts may be the site of ceramide transfer from its site of synthesis (ER) to sphingomyelin (SM) synthase via ceramide transfer protein (CERT). CERT extracts ceramide from the ER and transfers it to Golgi membranes. We have previously shown that CERT is enriched at the Golgi region and the Golgi localization of CERT puncta requires both ER and Golgi binding domains of CERT. We also showed that some Golgi structural perturbations reduced SM synthesis, suggesting that the organization of the mammalian Golgi ribbon together with CERT may promote specific ER-Golgi interactions for efficient delivery of ceramide for SM synthesis. We are currently examining the correlation between CERT localization and SM synthesis during proapoptotic stress. Our preliminary results suggest that newly synthesized SM levels and CERT localization are altered after treatment of cells with proapoptotic stimuli. We have also found that CERT is cleaved in cells treated with proapoptotic stimuli. In vitro, CERT is cleaved at a single site by caspase-2, a caspase that is partially localized to Golgi membranes. We are currently analyzing the effects of the caspase-2 generated CERT fragments on ceramide trafficking to gain further insight into the contribution of Golgi structure and Golgi localized caspase-2 pool to SM synthesis.
2199/B576
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To better understand the mechanisms and machinery that beta cells use to make and transport the hormone insulin to the cell surface for release after stimulation with glucose, we have used electron tomography to quantitatively map beta cell organization in 3D under different physiological conditions, at the nanometer scale. High (~5nm) resolution 3D reconstructions of the Golgi region in insulin-secreting cells have already provided a number of important insights regarding structure-function relationships among the key compartments (Golgi, mitochondria, insulin granules) involved in insulin production and release. To place our previous findings in context at the level of the whole cell, we recently developed a method for reconstructing mammalian cells in their entirety in 3D at intermediate (10-20nm) resolution. Here we report that through use of this ‘whole cell mapping’ approach we have been able to accurately map and relate changes in the distribution and size of organelles in beta cells to their relative level of biosynthetic/secretory activity. Our comparative analysis of four beta cells (two glucose-stimulated, two steady-state/non-stimulated) reconstructed in toto in 3D in this manner revealed that ‘resting’ (non-stimulated) beta cells demonstrated a significant degree of reproducibility in terms of the number, size and distribution of organelles. Likewise, the ratios determined for immature versus mature insulin granules for all four cells, when taken together with relative differences in surface area and volume measurements for the Golgi ribbon in general versus trans-Golgi cisternae, provided precise indicators of the level of biosynthetic activity for each of the cells. Quantification of relative differences in the structure and organization of the mitochondria themselves (number, length, extent of branching/fission/fusion) combined with significant differences in terms of their spatial distribution and extent of interaction with other compartments like the Golgi and insulin granules accurately reflected the relative energy needs of resting versus ‘active’ beta cells associated with glucose-stimulated (pro)insulin biosynthesis and secretory granule formation/release.

2200/B577
Modulation of Golgi Stacking and Cell Cycle Progression by Expressing Non-Regulatable Grasp65 Mutants in Mammalian Cells.
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The peripheral Golgi protein GRASP65 has been identified as a Golgi stacking factor in an In Vitro assay. Biochemical studies suggest that it links adjacent Golgi cisternae by forming mitotically regulated trans-oligomers. These conclusions, however, need further confirmation in the cell. In this study, we demonstrated that the N-terminal 112 amino acids (including the first PDZ domain) of the protein is sufficient for oligomerization. Systematic electron microscopic analysis results showed that the expression of non-phosphorylatable GRASP65 mutants in HeLa cells enhanced Golgi stacking in interphase and inhibited Golgi fragmentation in mitosis. Depletion of GRASP65 in HeLa cells by small interference RNA (siRNA) reduced the number of Golgi cisternae in the Golgi stack; this reduction was rescued by expressing exogenous rat GRASP65. These results provided evidence and a molecular mechanism by which GRASP65 stacks Golgi cisternal membranes. Further experiments also revealed that inhibition of Golgi disassembly during mitosis caused a delay in mitotic entry and suppressed cell growth, suggesting that Golgi disassembly at the onset of mitosis may play a role in cell cycle progression.
Endosomes and Lysosomes (2201 – 2215)

2201/B578
Rip11/FIP5 and Rab11 Binding to Sorting Nexin 18 Mediates Apical Endocytic Protein Sorting and Transport in Polarized Epithelial Cells.
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Epithelial cells are structurally and functionally polarized to transport specific molecules selectively and uni-directionally while maintaining trans-epithelial barrier. This selective transport is achieved by a junctional complex that partitions the plasma membrane into two distinct domains: apical and basolateral, with both of these plasma membrane compartments having distinct lipid and protein compositions. Apical transport is fundamental to the function of all epithelial cells and malfunctions in a number of diseases. Rab11 GTPases are members of the small monomeric GTPase super-family that have been implicated in regulating apical endocytic transport. In the last few years several Rab11-binding proteins have been identified, including the Rab11 family interacting proteins, also known as FIPs. In this study we show that Rip11/FIP5 is highly enriched at the apical pole of polarized MDCK cells as well as in the rat kidney tubules. Furthermore, we use MDCK cell line, stably-expressing tet-inducible Rip11/FIP5 shRNA, to demonstrate that Rip11/FIP5 plays a key role in protein transport from apical recycling endosomes to the apical plasma membrane, as well as, formation of apical lumen of epithelial cysts in 3D cultures. Work from several laboratories, including our own, have shown that FIPs act as scaffolding factors allowing the assembly of specific sorting/transport complexes required for the endocytic protein traffic. Thus, used proteomics to identify Sorting Nexin 18 (SNX18) as a Rip11/FIP5-binding protein. We have mapped Rip11/FIP5 and SNX18 binding motifs and shown that Rip11/FIP5 binding activates SNX18, thus promoting SNX18 oligomerization and induction of apical endocytic carrier formation In Vitro and in vivo. Consistent with that, we demonstrate that SNX18 mediates apical protein transport and is required for apical lumen formation of epithelial cysts in 3D cultures. Based on these data, we propose that the binding of Rab11 and Rip11/FIP5 protein complex to SNX18 activates SNX18-dependent budding/sorting of apical endocytic proteins carriers in polarized epithelial cells.

2202/B579
The V-ATPase Is Essential for Cellular Salt Detoxification.
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Vacuolar H+-ATPases generate proton gradients in endosomes and lysosomes. This is necessary for the uptake of different ions and metabolites into the yeast vacuole, which is analogous to the lysosome and is a major reservoir for these molecules. To find cellular processes that require V-ATPase function, we performed a genetic screen in Saccharomyces cerevisiae, looking for gene deletions that compromise growth in combination with V-ATPase deficiency. We found that V-ATPase subunits exhibit genetic interactions with members of the high osmolarity glycerol (HOG) pathway, which is activated by osmotic stress. Microarray analysis revealed that both positive and negative regulators of the HOG pathway are upregulated in a vma2 deletion mutant, suggesting that HOG pathway activation compensates for loss of V-ATPase function. Furthermore, V-ATPase deletion mutants have growth defects when exposed to high amounts of salt. This salt sensitivity is reflected by Hog1p activation at sub-saturating salt concentrations. Interestingly, vma2hog1 double deletion mutants exhibit a severe synthetic phenotype, characterized by slow growth and abnormally elongated cells. These double mutants are highly sensitive to salt stress, even more sensitive than mutants with single deletions of HOG1. Ste20p, a p21-activated kinase (Pak), activates several MAPK pathways in yeast that respond to pheromones, nutrient starvation, and osmotic stress. We show that the H subunit of the V-ATPase physically interacts with Ste20p using the split-ubiquitin system and In Vitro pull
down assays. Purified vacuoles from a ste20 deletion mutant have lower V-ATPase activity, while vacuolar vesicles from strains that over-express STE20 have increased activity. These results suggest that the V-ATPase acts in parallel with the HOG pathway in order to detoxify excess salt, and that Ste20p may be the link between V-ATPase function and the HOG pathway.

2203/B580

**Coordinated Dynein-Mediated Transport and Membrane Trafficking of Late Endocytic Organelles Is Essential for Proper Autophagy-Lysosomal Function in Neurons.**

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Lysosomes are dynamic organelles that receive and degrade macromolecules and organelles from secretory, endocytic, and autophagic pathways. Although some features of late endocytic trafficking have been described, regulation of this process in neurons remains to be elucidated. Here, we report an essential role for Snapin in regulating neuronal autophagy-lysosomal function by coordinating dynein-driven retrograde transport and late endocytic membrane trafficking. First, deletion of the snapin gene in mice results in impaired late endocytic transport and aberrant focal accumulation of late endosomes along neuronal processes, a phenotype that can be efficiently rescued by reintroducing the snapin gene into mutant neurons. Second, Snapin is relatively enriched in the late endocytic compartments and stabilizes dynein motor complex attached to late endocytic organelles. Third, snapin deficiency in mouse embryonic fibroblasts (MEFs) significantly results in impaired degradation of internalized EGF and BSA-gold, retention of internalized Dextran in late endosomes rather than delivery to lysosomes, and reduced maturation of lysosomal enzyme cathepsin D. Consistent with a defect in lysosomal degradation, an abnormal accumulation of autolysosome-like structures was observed in snapin-deficient hippocampal slices, cultured cortical neurons, and MEFs. Furthermore, we further demonstrated that elevated Snapin expression facilitates dynamic formation of tubular lysosomes by enhancing retrograde transport of late endosomes. Thus, using molecular and cellular approaches and time-lapse imaging in live cells combined with mouse genetic analysis and gene rescue experiments, our study provides mechanistic insights into the spatio-temporal coordination of late endocytic transport, membrane trafficking, and autophagy-lysosome function in neurons. Since impaired autophagy-lysosomal system has been implicated in neurodegeneration, Snapin-mediated up-regulation of autophagy-lysosomal function may provide a new regulatory pathway and possible therapeutic target to remove aggregation-prone intracytosolic proteins that are associated with a variety of neurodegenerative diseases.

2204/B581

**Caveolin Traffics to the Late Endosomal/Lysosomal Compartment.**

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Caveolae are endocytosed from the plasma membrane via caveolin coated vesicles, or cavicles, that travel to multiple destinations including caveosomes, the ER and lipid droplets. Here we identify another subcellular organelle to which caveolin traffics, the late endosome/lysosomal compartment (LE/lysosome). We show that caveolin traffics to these organelles when cells are starved in serum free medium, using several techniques, including live cell imaging and electron microscopy. Caveolin is bound to the external surface of lysotracker positive structures in starved cells since it is not degraded and dissociates within minutes upon the addition of fresh growth medium. Trafficking seems to be triggered by a change in pH, since the intra-lysosomal pH of starved cells is higher than that of cells maintained in growth medium. Trafficking appears to be blocked by specific drugs. Interestingly the structure of the caveolin under these conditions appears to be very different. Cavicles normally shuttle between membranes without an exchange of caveolin-1 and without a loss of
domain identity and caveolin appears as distinct puncta in the presence of the cholesterol trafficking drugs. However, when caveolin associates with LE/lysosomes during starvation the caveolin is evenly distributed over the lysosomal membrane surface suggesting that the structure of the cavicle/caveolae is significantly altered. It is well known that in the presence of cholesterol controls the shape of caveolae and when cholesterol is depleted the caveolae flatten into the membrane and caveolin is free to diffuse away. We suggest that the difference in the structure of the caveolin under these two conditions may reflect a difference in the cholesterol content of the lysosomal membrane. These findings lend further support to the idea that caveolin participates in intracellular cholesterol trafficking and suggests that it may be involved in cholesterol movement out of LE/lysosomes to either the ER or plasma membrane.

2205/B582
In Vitro Manipulation of Lysosomes Containing Fe$_2$O$_3$ Nanoparticles in HeLa EGFP-654 Cells.
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One of the challenges of non-viral gene delivery is the ultimate fate of the non-viral vector once it has crossed the initial cellular membrane. It has been shown that nanoparticles are first encapsulated into early endosomes, which follow the pathway to becoming lysosomes. In this study, we show the manipulation of lysosomes encapsulating paramagnetic nanoparticles in vitro. To this end, we have fabricated fluorescently labeled Fe$_2$O$_3$ nanoparticles and introduce them into the intracellular space of HeLa EGFP-654 cells using a permanent magnet. Using color channel co-localization of fluorescence images, we track the movements of fluorescently labeled lysosomes with encapsulated paramagnetic nanoparticles as they move through the cell interior. Subsequently, forces are exerted on these particles using a single pole 3DFM. We identify trends in the motions and mean square displacements of individual nanoparticles with a magnetic force and show that the motion of the nanoparticle is biased with respect to the behavior without an applied force. It is also shown that the motion of the manipulated nanoparticle containing lysosome is biased with respect to the "empty", non-manipulated lysosome. Our ultimate goal is to demonstrate the use of this manipulation in gene therapy, by showing delivery of a 20-base splice-correcting oligonucleotide.

2206/B583
Intracellular Localization of the Novel Polyglutamine Protein Kiaa1946.
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Kiaa1946 is a novel protein that contains a polyglutamine (polyQ) stretch within its primary amino acid sequence- polyQ tract proteins are particularly interesting because expansion mutation within them has been shown to underlie a growing list of severe neurodegenerative disorders such as Huntington’s Disease and Spinocerebellar Ataxia. Using a bioinformatics approach, we have found that Kiaa1946 is likely expressed in the nervous system, and contains a putative signal sequence and a single transmembrane domain. These data suggest that it likely plays a functional role in some aspect of the neuronal endomembrane system. As an initial attempt to ascertain which subcellular compartment Kiaa1946 functions in- we have cloned it into pEGFPN1, and assayed its intracellular localization in tissue culture cells using fluorescence microscopy. We find that Kiaa1946 displays a “vesicular” staining pattern, reminiscent of endosomes or lysosomes. However, colocalization studies using organelle specific antibodies suggest that Kiaa1946 is likely not in either of these two compartments. These data represent a first step in characterizing the cellular function of the novel polyQ containing protein Kiaa1946, and serve as a starting point for further investigations including: verification of subcellular
localization, protein interaction studies, and assays to test for polyQ polymorphism within the Kiaa1946 gene.

2207/B584

An SiRNA-Based Screen for Motor Proteins That Control Lysosomal Trafficking and Integrity in Cancer Cells.

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Tumor invasion and metastasis are associated with altered lysosomal trafficking and increased expression of the lysosomal proteases cathepsins. These changes may be necessary for cancer progression, but emerging experimental evidence suggests that they also render tumor cells more sensitive to lysosomal membrane permeabilization and subsequent cathepsin-mediated cell death. Thus, the lysosomal compartment is likely to be a good target for cancer therapy. The aim of this study is to identify proteins that control lysosomal trafficking and integrity in cancer cells in order to find new targets for cancer therapy. For that purpose we have designed an siRNA library in triplicate that target 146 known motor proteins (e.g. kinesins, dyneins and myosins). A screen for cell death has revealed nine motor proteins whose depletion by a minimum of two independent siRNAs results in at least 40% reduction in the survival of MCF-7 breast cancer cells and U2OS osteosarcoma cells. Some of the hits are involved in both vesicle trafficking and mitosis. However, a cell cycle status has revealed that only one hit, namely Kif11, induces accumulation in G2/M phase, suggesting that cells do not die from cell cycle inhibition. We are presently evaluating the cell death processes induced with particular focus on the involvement of lysosomal membrane permeabilization. Also, we are looking into changes of lysosomal trafficking prior to cell death.

2208/B585

CRESCent: Cluster of Recycling Endosomes Transiently Formed Around Spindle Pole Centrosome.

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Upon cell division, each organelle must be equally allocated into two daughter cells, and must therefore undergo dynamic morphological changes. For example, in interphase cells the Golgi apparatus is located in the perinuclear region, but it disassembles in prophase/prometaphase. In each daughter cell, two Golgi clusters are formed, near the spindle pole and the minus end of the central spindle in telophase; these join each other to reconstitute a single Golgi apparatus as cytokinesis proceeds. In contrast, behaviors of other organelles during cell division have been poorly characterized; endocytic compartments are thought to undergo ordered partitioning similar to that of the Golgi, although not precisely described. By following spatiotemporal changes in the distribution of EGFP-tagged transferring receptor, we discovered that recycling endosomes (REs) form a tight cluster around each spindle pole (CRESCent: Cluster of Recycling Endosomes around Spindle Pole Centrosome) in metaphase, long before Golgi reassembly. As cytokinesis proceeds, the CRESCent gradually disappears and REs reassemble near the spindle midzone. Closer inspection of the CRESCent structure revealed the presence of twin crescents around each spindle pole centrosome, and electron microscopy showed clustered vesicles containing internalized transferrin around centrosomes. FRAP analyses, however, revealed marginal membrane exchange between the CRESCent and other cellular compartments until anaphase/telophase, when membrane traffic becomes active. Our observations provide new insights into the relationship between assembly of REs around spindle poles and restriction of membrane delivery to the cell surface during mitosis.
Amyloid Precursor Protein and Its Shedding Enzyme, BACE1, Are Differentially Internalised and Sorted to Endosomal Compartments.

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There is increasing evidence that endosomes constitute a major site where Aβ peptides, the primary constituents of senile plaques which are a hallmark in the pathology of Alzheimer’s disease, are produced. Aβ peptide is generated by the sequential cleavage of the amyloid precursor protein (APP) by BACE1 and γ-secretase. Hence, factors that promote APP internalisation likely enhance Aβ production while blocking it decreases Aβ formation. Recently, enhanced BACE1 endocytosis was shown to equally promote Aβ production, although the molecular machinery involved in its internalisation remains elusive. Here we have analysed the internalisation route followed by BACE1. Transport of BACE1 is majorly governed through an acidic cluster-dileucine motif contained in its cytosolic tail that binds to GGAs. Since GGA3 was recently shown to be associated with the Arf6-dependent macropinocytotic route, we investigated the potential role of Arf6 in BACE1 trafficking. By using confocal microscopy we can demonstrate that BACE1 colocalises with both GGA3 and Arf6 at the cell surface. Moreover, overexpressing Arf6-Q67L, a dominant active form that prevents delivery of Arf6 cargo to endosomal compartments, entraps BACE1 in Arf6-Q67L vacuoles, and indicates that BACE1 utilises the Arf6-dependent internalisation route. BACE1 transport was also stalled with Arf6-T27N, a dominant negative form of Arf6 that blocks the recycling to the cell surface. Interestingly, when co-expressed, APP was only found to colocalise with BACE1 in Arf6-T27N overexpressing cells. Overexpression of these mutants resulted in respectively decreased and increased APP processing and Aβ secretion. Further analysis using antibody uptake and cell surface biotinylation in combination with overexpression of dominant negative mutants for clathrin-mediated internalisation clearly support our findings that BACE1 and APP can indeed enter the cell via distinct routes. These findings further underscore the importance of the (early) endosomal compartment as the major BACE1 processing compartment and open novel avenues for interfering with Aβ production through selectively interfering with the distinct internalisation routes.

The Calcium Channel MCOLN3 Regulates Endosomal Acidification.

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The cation channel mucolipin 3 (MCOLN3 or TRPML3) is one of the members of the mucolipin family. A punctual mutation in the mouse gene of MCOLN3 results in the varitint-waddler phenotype characterized by auditory and vestibular defects as well as pigmentation abnormalities. Recent observations showed a strong involvement of mucolipins in protein trafficking and recycling along the endo-lysosomal pathway. MCOLN3, suspected to play a role in melanosome trafficking, was recently shown to cause a dramatic alteration in the endosomal pathway when over-expressed. Indeed modification of the expression of MCOLN3 induces enlargement of HRS-positive endosomes, delays autophagosome maturation, and impairs degradation of epidermal growth factor (EGF) and EGF receptor. Calcium and pH play a crucial role in cargo trafficking, membrane fusion, and in the biogenesis and regulation of the endosomal machinery. Because MCOLN3 is a Ca²⁺-permeable channel regulated by pH, we hypothesize that MCOLN3 could couple pH and Ca²⁺ concentration along the endosomal pathway. To elucidate the function of MCLON3 and the consequences of its misexpression, we analyzed the outcomes of its over-expression, depletion or mutation on endosomal pH and Ca²⁺ concentration. Probes sensitive to pH (FITC-Dextran) or calcium (Oregon Green Bapta-5N) were internalized during 10 minutes in ARPE19 cells infected with virus encoding either the wild-type MCOLN3, its negative mutant, or a shRNA targeted against endogenous MCOLN3. Live cells were then processed by confocal microscopy to quantify pH and Ca²⁺ concentration along the endosomal pathway 20 minutes, 1 hour or 3 hours after internalization of the probe. Over-expression of wild-type MCOLN3, mutated MCOLN3, or depletion of the endogenous protein induced a dramatic
increase in both pH and Ca^{2+} concentration along the endosomal pathway. This study brings new insight in the mechanism of endosomal pH and calcium regulations, and in the involvement of MCOLN3 in this regulation. Further experiments, including In Vitro endosomal fusion assay, are ongoing to gain additional information about the function of MCOLN3 and the acidification and calcium regulation of the endosomal pathway.

2211/B588
Vacuole Size Scaling in Saccharomyces cerevisiae.
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Vacuoles in Saccharomyces cerevisiae perform a number of functions including waste processing and maintaining homeostasis. The morphology of organelles has direct implications for their function. for instance, the volume and surface area (measures of the lumen and membrane, respectively) of the vacuole affect its capacity to perform degradative functions and respond to external stress. Vacuoles in yeast grown to stationary phase are approximately spherical, and their surface area shows a linear scaling with respect to cell size. This result leads us to the preliminary hypothesis that vacuole surface area is controlled as a primary variable, and that volume is adjusted secondarily. To validate this concept, more detailed analysis is necessary to address more complex vacuole morphologies of yeast in growth phases. We present here a method for calculating surface area and volume of membrane-labeled vacuoles imaged with spinning disk confocal fluorescence microscopy. This method automatically determines the vacuole edges in three dimensions from which size parameters can be determined, and it is able to measure vacuoles with arbitrary shapes and number of subcompartments. Future experiments will characterize mutants with altered vacuole morphology to determine the principles behind the organelle's maintenance as well as how morphology affects function and fitness.

2212/B589
Phosphatidylinositol-4, 5-Bisphosphate 4-Phosphatase Interacts with MCOLN3 and Is Involved in the Trafficking of Cargo Proteins Along the Endo/Lysosomal Compartment.
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MCOLN3 is a Ca^{2+}-permeable channel that belongs to the family of transient receptor potential ion channels and is regulated by pH. In the varitint-waddler mice model, defects in pigmentation and deafness are linked to mutations in the MCOLN3 gene. Recently, we have shown that MCOLN3 localizes to early/late endosomes and is involved in protein trafficking along the endo/lysosomal pathway. To better understand the function of MCOLN3 we decided to perform a split-ubiquitin yeast-two hybrid screen using full length MCOLN3 as bait to search for potential partners. We identified an endosomal/lysosomal transmembrane protein named phosphatidylinositol-4, 5-bisphosphate 4-phosphatase (PI2P-4Pase). PI2P-4Pase catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-5-phosphate. We confirmed the interaction between GFP-MCOLN3 and HA-PI2P-4Pase by co-immunoprecipitation experiments. Using immunofluorescence and confocal microscopy we found that over-expressed HA-PI2P-4Pase extensively co-localized with GFP-MCOLN3 in CD63-/Lamp1-positive structures in ARPE19 cells. To study more in detail the trafficking of PI2P-4Pase we expressed either the catalytic or the transmembrane domain of PI2P-4Pase fused to GFP. The catalytic domain remained mostly in the cytosol whereas the transmembrane domain localized mainly to the plasma membrane and Golgi. We did not observed endosomal/lysosomal localization of these constructs suggesting that there is a trafficking motif within the catalytic domain of PI2P-4Pase. Amino acid sequence analysis of PI2P-4Pase revealed a putative dileucine-sorting motif (ERSP_{12}L_{13}) in its catalytic domain. Leucines substitution by alanines (ERSPA_{12}A_{13}) significantly increased the plasma membrane localization of HA-PI2P-4Pase suggesting that a fraction of PI2P-4Pase travels to the membrane before reaching the endosomal/lysosomal compartment. Interestingly, over-expression of GFP-PI2P-4Pase affected trafficking of endocytosed Epidermal Growth Factor Receptor (EGFR). Western blot analysis of ARPE19 cells over-expressing GFP-
PI2P-4Pase showed delayed degradation of EGFR. All together, our data show that PI2P-4Pase is involved in protein trafficking along the endo/lysosomal pathway.

2213/B590
The Connexdenn 1 DENN Domain: A GEF for Rab35 Mediating Cargo-Specific Exit from Early Endosomes.

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Abstract: The DENN domain is an evolutionary ancient protein module conserved from yeast to humans. Mutations in the module cause developmental defects in plants and human diseases, yet the function of this common domain remains unknown. We now demonstrate that the DENN domain of connexdenn 1, which we originally identified based on its enrichment on clathrin-coated vesicles (Allaire et al., J. Neurosci., 2006), binds to Rab35 and recruits this GTPase to clathrin-coated pits and vesicles. Connexdenn 1 remains associated with the vesicles following uncoating, allowing the protein to recruit Rab35 to the endosomal system. Moreover, we demonstrate that the DENN domain of connexdenn 1 functions as a guanine nucleotide exchange factor to regulate Rab35-mediated endosomal trafficking. Surprisingly, loss of function of connexdenn 1 or Rab35 did not influence clathrin-mediated endocytosis of transferrin or its recycling. In contrast, loss of Rab35 activity inhibits MHCI recycling and prevents the early endosomal recruitment of EHD1, a common component of endosomal recycling tubules. Our data are the first to reveal an enzymatic activity for a DENN domain and demonstrate that distinct Rab GTPases can recruit a common protein machinery to various sites within the endosomal network to establish cargo-selective recycling pathways.

2214/B591
Interaction of the Salmonella Containing Vacuole with Lysosomal Hydrolases and the Retromer Complex.

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Salmonella enterica serovar Typhimurium is an intracellular pathogen that resides within a modified phagosome called the Salmonella containing vacuole (SCV). Invasion of nonphagocytic cells and subsequent biogenesis of the SCV, in both phagocytic and nonphagocytic cells, are dependent on a number of bacterial effector proteins that are “injected” or translocated into the host cell via two Type III Secretion Systems (T3SS). T3SS1 is required for invasion and is also implicated in the initial SCV biogenesis. In contrast T3SS2, which is induced following invasion, is required for later stages in SCV biogenesis and particularly the formation of an extensive tubular network known as Sifs (Salmonella-induced filaments) in infected epithelial cells. Biogenesis of the SCV and Sifs is characterized by the acquisition of lysosomal membrane proteins, such as LAMP1 and LAMP2, but the question of whether or not SCVs/Sifs fuse directly with lysosomes remains controversial. Several studies have shown that the mannose 6-phosphate receptor (M6PR) and the hydrolases that it delivers to lysosomes, are excluded from SCVs leading to the conclusion that SCVs avoid fusion with lysosomes. Other studies, however, have shown that SCVs can fuse directly with lysosomes and that consequently lysosomal content markers, such as fluorescent dextrans, are delivered to the lumen of the SCV. To gain a better understanding of how Salmonella establishes its intracellular replicative niche in epithelial cells, and in particular how it avoids the antibacterial activities of lysosomes, we are investigating the trafficking and activation states of lysosomal hydrolases in HeLa cells infected with S. Typhimurium. Live cell imaging, using a fluorescent cathepsin-B probe, revealed the presence of active hydrolase within the SCV at 2 hr post infection. We also found that depletion of components of the retromer complex (Snx-1, Rab7) resulted in enrichment of M6PR on the SCV. Our data suggest that active
hydrolases are present in SCVs and that, similar to lysosomes, M6PR is rapidly removed from the SCV via a retromer-dependent process.

2215/B592
Characterization of Two Novel Yeast Mutants Defective at Endosome and Vacuole Interface -- env6Δ and env7Δ. M. Serranilla, D. K. Olson, C. Aguilera, F. Ricarte, Q. Nguyen, T. Soreta, E. Gharakhanian; Biological Sciences, CSULB, Long Beach, CA

The vacuole of the fungus *Saccharomyces cerevisiae* has been a seminal model for the studies of lysosomal trafficking, biogenesis, and function. Our laboratory previously uncovered 15 deletion mutants associated with these processes utilizing a genome-wide immunodetection screen of a MAT-α deletion strain library of non-essential genes. This study focuses on the characterization of two of the uncovered and previously uncharacterized endosome and vacuole interface (*env*) mutants- *env6Δ* and *env7Δ*. Microscopic, biochemical, and bioinformatics techniques were used in the characterization of the two mutants with respect to vesicular trafficking, vacuole morphology, growth under various conditions associated with vacuolar defects, and gene homology. *env6Δ* contains fragmented vacuoles with acidification defects and exhibits severe growth sensitivities to a variety of conditions tested. *ENV6* encodes a putative transmembrane protein of 102 amino acids and appears to be conserved only in closely related fungi. *env7Δ* exhibits normal vacuolar morphology with no severe sensitivities to various growth conditions. *ENV7* encodes a 364 amino acid protein which contains a serine/threonine kinase domain with 29% identity to the human/mammalian myristylated and palmitoylated STK16 kinase.

ER to Golgi to Cell Surface (2216 – 2231)

2216/B593
Modulation of Angiotensin II Type 2 Receptor Transport from the Endoplasmic Reticulum to the Cell Surface By the Diacidic Exd Motif in the C-Terminus.
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The physiological function of angiotensin II (Ang II) is mediated through the Ang II type 1 (AT1R) and type 2 receptor (AT2R), which belong to the superfamily of seven transmembrane cell surface receptors coupled to heterotrimeric G proteins. We have recently demonstrated that AT1R exit from the endoplasmic reticulum (ER) is directed by highly conserved single L residue in the first intracellular loop and F(x)6LL motif in the C-terminus. In contrast, the structural determinants for angiotensin II type 2 receptor (AT2R) export trafficking remain undefined. In this study, we have demonstrated that the AT2R mutant lacking the entire C-terminus was unable to transport to the cell surface. Progressive deletion of the C-terminus and alanine-scanning mutagenesis identified two acidic residues, E357 and D359, in the C-terminus, which are required for AT2R exit out of the ER and transport to the cell surface. Mutation of E357 to a or D, D359 to a or E, ExD to AxA, and ExD to DxE significantly attenuated AT2R transport from the ER to the cell surface in HEK293 and PC12 cells. The mutated AT2R tagged with either GFP or HA was extensively localized within the ER. In contrast, these mutations and deletions did not alter total AT2R expression. These data demonstrate that the diacidic ExD motif plays an important role in modulating export trafficking of newly synthesized AT2R from the ER to the cell surface.

2217/B594
Mechanisms of Retention and Transport of the ER-Stress Sensing Transmembrane Transcription Factor CREB-H.
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CREB-H is an ATF6-related transmembrane transcription factor which in response to ER stress, traffics to the Golgi where it is cleaved by specific proteases, S1P and S2P, to produce the active N-terminal domain that travels to the nucleus to effect appropriate transcriptional responses. While the broad regulatory pathways are similar, CREB-H appears to sense and regulate distinct ER-related stress and appropriate responses, but as yet little is known about the mechanisms or determinants involved. We demonstrate that unlike in ATF6 whose luminal tail binds BiP and contains determinants for stress sensing and Golgi transport, in CREB-H the luminal tail is not involved in ER retention, not required for Golgi transport and does not bind BiP. The main determinant for CREB-H ER retention resides in a novel membrane-proximal cytoplasmic determinant that is conserved in related members of the CREB-H family, but lacking in ATF6. We refine requirements within the ER-retention motif (the ERM) and show that ERM-ve variants exhibited constitutive Golgi localisation and constitutive cleavage by the Golgi protease, S1P. The ERM also confers ER-retention on a heterologous protein. Furthermore deletion of the luminal tail of CREB-H had no effect on ER-retention of parental CREB-H or Golgi localisation of ERM-ve variants. Importantly, when the luminal tail of ATF6 was transferred into an ERM-ve variant, the chimera was now retained in the ER, and was responsive to chemical inducers of UPR. Together these data demonstrate novel and qualitatively distinct mechanisms of trafficking and stress signaling in CREB-H compared to ATF6 and identify a key determinant through which CREB-H ER stress sensing and transport may be regulated.

2218/B595
Regulation of Components of the Secretory Pathway and Solute Transporters by the ER-Stress Responsive Transmembrane Transcription Factor CREB-H.
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Regulated intramembrane proteolysis and activation of ER-localised transcription factors, such as SREBP, ATF6 and the CREB3 family represents a key component in dealing with stress at the ER. These factors are controlled by ER-retention and stress-induced release/Golgi transport, resulting in site-specific cleavage by Golgi-proteases, producing the N-terminal active transcription factor. While the related factor ATF6 regulates genes involved in protein folding and assembly, for CREB-H, pro-inflammatory cytokines such as IL6 and TNFα have been reported to elicit CREB-H cleavage, resulting in up-regulation of certain acute-phase response genes. Nevertheless the repertoire of target genes and precise role(s) of CREB-H remain to be established. Here we develop an inducible system for the regulated induction of the active nuclear form of CREB-H. The protein is subject to phosphorylation and rapid turnover by F-box factors. Microarray analysis of target genes demonstrates a selective induction of genes involved in flux through the secretory pathway including among others KDEL receptors, SEC components, ARFGAP, together with two other groupings in solute transporters and genes involved in modification of extracellular components. Remarkably while induction of CREB-H slowed cell growth, cells became resistant to serum-withdrawal induced detachment from the substratum. Indeed CREB-H induction after cell detachment and aggregation, resulted in reattachment and organised growth and morphology. Consistent with conservation of features of the DNA binding domain CREB-H target genes exhibit overlap with those for the related factor CREB4, but are distinctly different from those of ATF6. These factors may be involved in collaboration with ATF6 in coupling proper flux through the secretory pathway with protein folding, in response to ER stress.

2219/B596
The SEC23-SEC31 Interface Is Critical for Procollagen Secretion.
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COPII proteins play an essential role in exporting most of the secretory proteins from the endoplasmic reticulum (ER). These coat proteins generate transport vesicles of approximately 80nm diameter that package variety of cargo molecules. It remains unclear how these small vesicles can accommodate large cargo molecules such as lipidated lipoproteins and procollagen which has the size of greater than 200nm. One possibility is that unusual COPII cage structures accommodating large cargo molecules exist. We originally described F382L SEC23A mutation resulting in a novel autosomal recessive craniofacial disease, cranio-lenticulo-sutural dysplasia (CLSD or Boyadjiev-Jabs syndrome). Prominent cellular features of skin fibroblasts from CLSD patients include a secretion defect resulting in significant distension of the ER. Recently, we have identified heterozygous M702V SEC23A mutation in an unrelated patient with clinical features of CLSD. This M702 residue is highly conserved and localized in the SEC23-SEC31 interface. Interestingly, this patient has inherited the M702V allele from his clinically unaffected father. No maternal SEC23A mutation was identified. Cultured skin fibroblasts from this patient showed a secretion defect of procollagen and ER distension. We suggest that this case may manifest digenic inheritance and the maternal mutation may involve another COPII member or a respective cargo molecule. Consistent with this hypothesis, we observed excessive retention of procollagen in the patient fibroblasts, while both parental fibroblast cell lines showed mild procollagen accumulation. Purified recombinant M702V SEC23A was able to normally bind SEC31 and to package small cargos such as ERGIC53. Our data suggest that even mutations with a mild effect at the SEC23-SEC31 interface may cause selective inhibition of large cargo export from the ER. This may indicate that optimal SEC23-SEC31 interaction is critical for assembly of COPII cage structures capable of accommodating large cargo molecules.

2220/B597
mTrs130 Is a Component of a Mammalian TRAPPIi Complex, a Rab1 GEF That Binds to COPI Coated Vesicles.
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The GTPase Rab1 regulates ER-Golgi and early Golgi traffic. The guanine nucleotide exchange factor (GEF) or factors that activate Rab1 at these stages of the secretory pathway are currently unknown. Trs130p is a subunit of the yeast TRAPPI complex, a multisubunit tethering complex that is a GEF for the Rab1 homologue Ypt1p. Here we show that mammalian Trs130 (mTrs130) is a component of an analogous TRAPP complex in mammalian cells, and describe for the first time the role that this complex plays in membrane traffic. mTRAPPI (mammalian TRAPPIi) is enriched on COPI coated vesicles and buds, but not Golgi cisternae, and it specifically activates Rab1. Additionally, we find that mTRAPPI binds to γ1COP, a COPI coat adaptor subunit. The depletion of mTrs130 by shRNA leads to an increase of vesicles in the vicinity of the Golgi and the accumulation of cargo in an early Golgi compartment. We propose that mTRAPPI is a Rab1 GEF that tethers COPI coated vesicles to early Golgi membranes.

2221/B598
The Role of Phosphorylation of a COPII Coat Protein, Sec31 in Molecular Export from the Endoplasmic Reticulum.
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Molecular export from the ER is thought to be mediated by COPII vesicles. It has been shown that an essential COPII coat component, Sec31 in yeast, is phosphorylated and its phosphorylation has been implicated in COPII vesicle budding. However, the molecular mechanisms, including the phosphorylation sites and the kinases for this phosphorylation, are
unknown. Hypothesizing that the phosphorylation-dephosphorylation cycle of the coat components of COPII vesicles may regulate molecular export from the ER, we recently discovered the phosphorylation of mammalian Sec31. Here we show that membrane recruitment of Sec31 is dependent on its phosphorylation. This suggests that the molecular export from the ER may be related to the phosphorylation of Sec31 in mammalian cells.

2222/B599
Rab1-Mediated Pro-Collagen Trafficking in Differentiating Osteoblasts.
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Collagen is the most abundant protein in mammals, and osteoblast bone cells are one of the chief collagen-producers in the body. Despite advances in investigating extracellular collagen assembly, there has been little research conducted on intracellular processes undermining pro-collagen transport in osteoblasts. Rab GTPases are critical regulators of vesicle trafficking in cells. We, therefore, hypothesized that Rab proteins are involved in transportation of pro-collagen vesicles from ER to Golgi to the PM. Rab expression at the mRNA levels was studied in differentiating osteoblasts using a microarray approach and Rab1 was found to be upregulated compared to undifferentiated control cells. To study the interaction of Rab1 GTPases with pro-collagen vesicle synthesis and transport, control and differentiating MC3T3 osteoblasts were co-transfected with procollagen-RFP and Rab1-EGFP as well as the dominant negative form of Rab1, and collagen trafficking was visualized using live cell epifluorescent microscopy. The transfected cells were also fixed and immunostained for endogenous intracellular and extracellular collagen and imaged using epifluorescent and TIRF microscopy. Our results thus far confirm the importance of Rab1 in pro-collagen trafficking from ER to Golgi in osteoblasts as Rab1-EGFP co-localizes with collagen. Furthermore, cells expressing the dominant negative mutant form of Rab1 show a significant reduction of intracellular collagen within biosynthetic organelles of the osteoblast.

2223/B600
EXP1: A Novel Adaptor Involved in Trafficking of Membrane Proteins from the ER in S. Cerevisiae.
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The plasma membrane H+ ATPase (Pma1p) is the most abundant protein in the plasma membrane of S. cerevisiae and is a major cargo of the secretory pathway. Our lab previously showed that Lst1p, a homolog of Sec24p, is needed for efficient COPII trafficking of Pma1p out of the ER. An lst1Δ is viable but exhibits sensitivity to growth in acidic medium due to a decrease in Pma1p at the plasma membrane. We have now identified a novel gene, named EXP1 (ER export of Pma1p), that when overexpressed restores Pma1p plasma membrane localization in an lst1Δ. A deletion of EXP1 is viable, but an lst1Δ exp1Δ double mutant is inviable and displays a severe Pma1p trafficking defect. There appear to be two pathways for Pma1p export from the ER; one mediated by Lst1p and another mediated by Sec24p. Exp1p is essential for function of the Sec24p pathway but not the Lst1p pathway. Exp1p is a 17 kD integral membrane protein with an N-terminal transmembrane domain and a highly charged cytosolic C terminus. Exp1p fractionates primarily with the ER in wild type cells and with the Golgi in COPI mutants, indicating that Exp1p cycles between the ER and Golgi. Recombinant Exp1p binds to Sec23/24p in vitro. We have identified short cis-acting sequences in the cytosolic C terminus of Exp1p that are required for export from the ER and interaction with Sec23/24p as well as retrieval from the Golgi, all of which are essential for Exp1p function. Thus, Exp1p appears to be required for transport of Pma1p from the ER via a Sec24p-mediated pathway. We are currently investigating the interactions between Exp1p and Pma1p. Exp1p represents a novel class of cargo adaptors that assist the loading of membrane proteins into Sec24p-coated vesicles.
2224/B601
A Novel Role for Sec24p during COPII Vesicle Biogenesis.
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Generation of ER-derived COPII transport vesicles requires the small GTPase Sar1p, the Sec23/24p adaptor layer, and the Sec13/31p outer coat. Sec23p serves as the GTPase activating protein (GAP) for Sar1p whereas Sec24p binds cargo proteins to promote efficient packaging into COPII vesicles. Here we describe the characterization of a novel mutation in Sec24p, sec24-A11, that provides evidence for an additional role in stabilizing the COPII coat during early vesicle formation through interaction with the large ER-bound scaffolding protein, Sec16p. Biochemical analysis reveals that the A11 mutation causes a general secretory delay and impairs GTP-dependent COPII budding despite normal COPII coat assembly in the presence of the non-hydrolyzable GMP-PNP. We implemented a synthetic dosage lethality screen to identify strains that are highly sensitive to overexpression of this mutant form of Sec24p and found that overexpression of Sec24p-A11 was detrimental in a sed4Δ strain. Sed4p is an ER-localized membrane protein that shows genetic interactions with SEC23 and SAR1, and also has genetic and physical interactions with SEC16, although the function of Sed4p is unknown. Further genetic studies revealed that sec24-A11 is synthetic lethal with the sed4Δ mutation, as well as with a temperature sensitive sec16-2 allele. Since Sec16p is a common interacting partner of both Sed4p and Sec24p, we examined whether the interaction between Sec16p and Sec24p is changed in the presence of the A11 mutation. Yeast-two-hybrid experiments demonstrated that Sec24p-A11 is unable to interact with Sec16p. Our findings on this new function of Sec24p will lead to a better understanding of the cooperative efforts of the COPII coat proteins and Sec16p in coordinating events during the production of COPII vesicles.

2225/B602
Expression of Gint3, the Drosophila Homolog of UBXD1, in Drosophila Embryos.
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UBX proteins contain a ubiquitin-like UBX domain and a glycanase-like PUG domain. UBX proteins interact with p97, an AAA ATPase. The functions of UBX proteins and the consequences of their interaction with p97 are not known. One hypothesis is that UBX proteins serve as linker proteins that recruit client proteins for the chaperone or proteasome targeting functions of p97. One possible client protein is rab GDP dissociation inhibitor (GDI), which our lab has found interacts with Gint3, the Drosophila UBXD1 homolog. The interaction has been shown in a yeast two hybrid system, in pull-downs of bacterially expressed proteins and in interaction of tagged proteins in transgenic flies. This result shows that Gint3 can bind to rab-free GDI, but it is not known whether Gint3 can bind to rab-bound GDI with the same affinity. To learn more about the possible range of functions of Gint3, we have studied its expression in flies. First, expression of Gint3 RNAi in transgenic flies was found to be lethal, supporting the idea that Gint3 function is essential for life in flies. Second, in situ hybridization studies found that Gint3 mRNA is shows a broad cytoplasmic staining in early embryos, with a possible elevation in the embryonic nervous system and mesoderm at mid-embryogenesis. This finding suggests that Gint3 may have special functions related to neurogenesis. To find out whether Gint3 binds to predominantly rab-free GDI or rab-bound GDI and what other proteins Gint3 may bind to, we have tagged Gint3 with epitope tags and transformed it into flies.

2226/B603
Alpha-Synuclein Delays ER-To-Golgi Transport in Mammalian Cells by Antagonizing ER/Golgi SNARES.
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Toxicity of human alpha-synuclein A53T when expressed in simple organisms can be suppressed by over expression of Endoplasmic Reticulum(ER)-to-Golgi transport machinery, suggesting that inhibition of constitutive secretion represents a fundamental cause of the toxicity. Whether inhibition of similar steps in the mammalian secretory pathway represents a fundamental cause of familial Parkinson’s disease has not been established. We tested elements of this hypothesis in mammals by expressing human alpha-synuclein in normal rat kidney cells and assessing ER-to-Golgi transport using quantitative fluorescence microscopy. Over expression of wildtype or A53T alpha-synuclein delayed transport by up to 50%, however, A53T inhibited more potently. The secretory delay occurred at low expression levels and was not accompanied by formation of large alpha-synuclein inclusions nor mistargeting of transport machinery to alpha-synuclein aggregates, suggesting a direct action of soluble alpha-synuclein on trafficking proteins in situ. Co-over expression of either the ER/Golgi R-SNARE sec22b, or the multifunctional R-SNARE ykt6 specifically rescued transport, indicating that alpha-synuclein expression antagonizes SNARE function. Ykt6 reversed alpha-synuclein inhibition much more effectively than sec22b, the native ER/Golgi R-SNARE, suggesting a possible neuroprotective role for the enigmatic high expression of ykt6 in neurons. In addition, we report that soluble alpha-synuclein A53T directly binds ER/Golgi SNAREs and inhibits fusogenic SNARE complex assembly, providing a potential mechanism for toxic effects in the early secretory pathway.

2227/B604
Regulation of Membrane Targeting to the Cilium by a Multimeric Complex Organized by Arf and Rab GTPases.
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We have recently shown that Arf4, which binds to the VxPx ciliary targeting motif present in rhodopsin functions within a novel trafficking module comprised of two small GTPases, Arf4 and Rab11, the Rab11/Arf effector FIP3, and the Arf GTPase-activating protein (GAP) ASAP1 (Mazelova et al., 2009, EMBO J. 28:183). In this study we examined the molecular architecture of the Arf4/Rab11/FIP3/ASAP1 complex. We studied the spatio-temporal interactions among the components of the complex, and with the ciliary cargo, rhodopsin, during In Vitro budding of rhodopsin transport carrier (RTCs). Targeting complex bound rhodopsin at the TGN. Arf4 was absent form budded RTCs, as reported before. Rab11/FIP3/ASAP1 were present on RTCs and remained in a complex, but this complex was no longer associated with rhodopsin. ASAP1 BAR-PZA, a recombinant protein containing the BAR, PH, GAP and ankyrin repeat domain of ASAP1, which stimulates RTC budding, bound purified rhodopsin directly. ASAP1 BAR-PZA, but not the PZA domain (lacking the BAR domain), specifically precipitated rhodopsin in the presence of GTP-Arf4, which suggests the formation of a tripartite complex. To demonstrate that the Arf4/Rab11/FIP3/ASAP1 targeting module is conserved in polarized cells containing primary cilia we generated IMCD-3 cells expressing a fusion protein comprised of bovine rhodopsin and eGFP spliced within the C-terminal tail, leaving the VxPx targeting signal available for molecular interactions. The VxPx motif correctly targeted rhodopsin::eGFP, which co-localized with acetylated α-tubulin in the cilia of cultured cells where it appeared to be most concentrated at the distal cilia tip. In ASAP1 siRNA transfected cells cilia stained with acetylated α-tubulin appeared normal, but lacked the eGFP-tagged rhodopsin. Our data support the notion that the Arf4/Rab11/FIP3/ASAP1 targeting complex has a general role in the selection and packaging of membrane cargo destined for delivery to primary cilia.

2228/B605
Does Cholesterol Traffic from the ER to the Plasma Membrane by a Golgi-Independent Pathway?
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Current theories of membrane traffic assert that cholesterol, unlike glycerophospholipid, is transported to the plasma membrane from its site of synthesis in the endoplasmic reticulum (ER)
by a process that is largely independent of the Golgi apparatus. This widely accepted theory relies on beautiful classical radiotracer experiments that may not be as definitive as current dogma implies. We adopted a systems biology approach using our ProcessDB software to test the alternative hypothesis that cytosolic lipid transfer proteins (LTP) could actually be responsible for the net retrograde transport of cholesterol from plasma membrane to ER and still be consistent with the classical ³H-acetate experiments. We formulated these hypotheses as computer models and used ProcessDB to test them against the classical experiments. With LTP binding constants in the published range, we found that it is entirely possible to account for the classical tracer kinetic data with a theory in which the net transport of cholesterol via LTPs is carried from the plasma membrane to the ER. Thus, it remains possible that the secretory pathway is responsible for the net anterograde cholesterol traffic to the plasma membrane from the ER.

2229/B606
Factors and Signals in TGN to Late Endosome Transport.
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In the budding yeast Saccharomyces cerevisiae, the trans-Golgi network (TGN) membrane proteins, such as the proprotein processing enzymes Kex2p and Ste13p, the cargo-sorting receptor Vps10p and components of the vesicle fusion machinery, require continual cycling between the TGN and the late endosome/prevacuolar compartment (PVC) for proper function and stability. We are using a cell-free, biochemical assay to dissect the molecular events of trafficking from the TGN to PVC. Our published work has shown that In Vitro transport requires clathrin heavy chain, the clathrin adaptor Gga2p, the dynamin homolog Vps1p, the Vps21p rab, the Vps45p SM protein and the PVC t-SNARE Pep12p. The system measures transport of the processing protease, Kex2, relying either on its own C-terminal cytosolic tail or that of the Vps10p sorting receptor, from the TGN to PVC membranes containing a recombinant Kex2 substrate, PSHA. Directed yeast two-hybrid and biochemical assays demonstrate that the VHS domain of Gga2p binds to sequences in the Kex2p tails. Gga2p binds to a novel sequence in the Kex2p tail that includes an essential Ser residue (780). Binding is seen to a tail sequence containing the phosphomimetic substitution to Asp 780, but not an Ala780 substitution. The Asp780 and Ala780 substitutions have opposite effects both on localization of Kex2p In Vivo and on transport of Kex2p in the cell free assay. An affinity-purified anti-phosphopeptide antibody recognized Kex2p but not Kex2p Ser780Ala, demonstrating the phosphorylation of this residue in vivo. Previously we found that the Soi1/Vps13 protein, an unusual 3144 residue novel protein, which has at least two human disease gene homologues, functions in both anterograde and retrograde transport from the TGN to the PVC. The In Vitro TGN-PVC assay requires Vps13p function and provides a complementation assay for purification of the functional molecule. In addition, we have identified yeast Centrin (Cdc31p) as a Vps13 binding protein. We are currently investigating the role of Cdc31p as well as yeast Arf1p and Arf2p GTPase in the In Vitro TGN-PVC transport reaction.

2230/B607
Arf6 Regulates Secretory Granule Trafficking in Neuronal Cells
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ADAP1 (centaurin alpha-1) is an Arf GAP expressed in neurons and neurosecretory PC12 cells. Previously, we demonstrated that ADAP1 localizes to immature secretory granules in neuronal cells. Moreover, manipulation of ADAP1 levels affects the rate of trafficking of chromogranin B, a regulated secretory granule cargo, in PC12 cells. As ADAP1 is an Arf6 GAP, we examined whether Arf6 has a role in secretory granule trafficking as well. In both PC12 cells and neurons, endogenous Arf6 localized with endosomal markers, as expected since Arf6 has been shown to function in endosomal trafficking. In addition, significant levels of colocalization were observed between endogenous Arf6 and the secretory granule markers, chromogranin a and B and
secretogranin. Arf6 also colocalized with furin, a peptidase localized to immature secretory granules. We next manipulated Arf6 levels using transfection of Arf6 mutants and siRNA in PC12 cells and used a temperature sensitive GFP-tagged chromogranin B cargo to monitor the rate of trafficking from the Golgi to the periphery. Expression of the GDP-locked Arf6 mutant, T27NArf6, or siRNA mediated knock down of Arf6, led to a significant inhibition of chromogranin B trafficking from the TGN. These data suggest a role for Arf6 in secretory granule trafficking, and are consistent with ADAP1 functioning with Arf6 to regulate secretory granule trafficking in neuronal cells.

**2231/B608**

**Domain Cooperativity Ensures Compartment Specificity and Cargo Selectivity of Myristoylated Naked2.**

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Naked2 is a 451 amino acid myristoylated protein that selectively recognizes TGFα-containing exocytic vesicles, but how vesicle recognition (VR) is achieved is unknown. Here we show that residues 1-36 of Naked2, including myristoylation, are necessary and sufficient for VR. Naked2 N1-36 contains dominant-acting VR based upon its ability to redirect NHERF1 from the cytoplasm to vesicles. However, in the context of full-length Naked2, VR can be achieved by N1-36 plus myristoylation or N1-36 lacking myristoylation plus the TGFα tail binding (TTB) domain. Wild-type Naked2 selectively binds phosphoinositide PI(4,5)P2 and this selectivity is lost with mutations in the VR domain. These results support a model in which cooperativity among individual weakly acting elements within Naked2 (myristoylation, VR domain, cargo and PI binding) ensures compartment specificity and cargo selectivity. Moreover, in addition to “myristate plus basic”, the N-termini of myristoylated proteins may contain “myristate plus compartmentalization” signals, a finding with implications for trafficking of myristoylated proteins.

**Membrane Domains and Polarity (2232 – 2246)**

**2232/B609**

**Correlation Function Analysis Corrects Artifactual Self-Clustering and Reveals Significant Co-Localization of FcεRI and Lyn in resting RBL-2H3 Mast Cells.**

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We use pair auto- and cross-correlation functions to quantify lateral heterogeneity within the plasma membranes of intact RBL-2H3 mast cells. Gold-antibody conjugates are used to specifically label plasma membrane proteins and lipids and are visualized using scanning electron microscopy with backscatter detection. An automated image-processing algorithm identifies positions of gold particle centers, enabling the processing of large datasets with high particle densities. Consistent with previous studies, we find that gold particles labeling a variety of plasma membrane lipids and proteins are highly clustered in resting cells. In contrast to previous studies, we find that this apparent clustering can be accounted for by multiple gold particles binding to single target proteins with Gaussian-shaped binding surfaces. This is demonstrated by imaging antibodies covalently conjugated to a silicon surface, by comparing correlation functions for a wide range of cell surface labels with varying surface densities, and by measuring cross-correlations between identical but distinguishable pools of either IgE-FcεRI or GM1 labeled with cholera toxin B on the cell surface. After correcting for artifactual clustering, we find that all (>5) proteins and lipids examined are not auto-correlated in resting cells at physiological temperatures, within experimental error bounds. In contrast, we find significant cross-correlation between IgE-FcεRI and the inner leaflet signaling protein Lyn in these unstimulated cells, and this co-clustering is only moderately modulated when membrane cholesterol levels are altered with
MβCD. Our correlation function particle distribution approach is likely to have wide applicability in nanoscale image analysis.

2233/B610
Mechanisms Regulating the Cell Surface Dynamics of the Lipid Raft Marker Cholera Toxin B Subunit.
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The B subunit of cholera toxin (CTXB) is generally accepted as a marker of lipid rafts. Compared to other raft markers or lipid-anchored proteins, CTXB exhibits relatively slow diffusion. A variety of mechanisms could potentially account for this slow diffusion of CTXB, including crosslinking of small raft domains, confinement by the actin cytoskeleton, association with caveolae, incorporation into actively maintained domains, or molecular crowding effects in response to elevated membrane protein density. We evaluated the role of each of these mechanisms in controlling the lateral diffusion of CTXB in the current study by employing fluorescence recovery after photobleaching (FRAP) of fluorescently labeled CTXB following actin depolymerization, ATP depletion, cholesterol depletion, labeling across a range of CTXB concentrations, or in caveolin-1 knockout MEFs. Of these conditions, only cholesterol depletion significantly altered the diffusional mobility of CTXB. Furthermore, we tested whether the slow diffusion of CTXB is an intrinsic property of its receptor by examining the effects of CTXB on the diffusion of a fluorescent GM1 analog. The results of this experiment showed that CTXB slows the diffusion of its receptor. However, binding of CTXB to cells did not affect the diffusion of another raft marker (YFP-GL-GPI), a non-raft marker (YFP-GT46), or a fluorescent lipid analog (DiIC16). Taken together, these data suggest that CTXB diffusion is not limited by actin corrals, caveolae, molecular crowding effects, or the intrinsically slow diffusion of GM1. In addition, they suggest that crosslinking of small rafts induced by CTXB binding does not substantially alter the dynamics of membrane domains enriched in other types of raft or non-raft proteins or lipids in this cell model.

2234/B611
Prominin-2 Positive Protrusions Colocalize With Lipid Raft Markers and Compete With Caveolin-1 for Plasma Membrane Cholesterol.
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Background & Objectives: Fluorescent lipid analogs have been used to probe the organization of plasma membrane (PM) lipids and to study the processes of endocytosis, sorting, and recycling of lipids. Interestingly, a fluorescent analog of lactosylceramide, Bodipy-LacCer, does not distribute uniformly over the cell surface, but rather is concentrated in micron size “domains” at the PM. The goal of this study was to determine the relationship of these domains to membrane protrusions. Methods & Results: (i) Human skin fibroblasts were transfected with a fluorescent chimera of prominin-2 (Prom2-mKate), a cholesterol binding protein that is confined to microvilli, cilia, and other acetylated tubulin-positive structures, to mark membrane protrusions. Cells were subsequently incubated with Bodipy-LacCer or -GM1 ganglioside to label the PM and visualized by fluorescence microscopy. Both lipid analogs co-localized extensively with Prom2-mKate protrusions. Extensive co-localization of Prom2-mKate with cholera toxin B (binds to endogenous GM1 ganglioside) and PEG-cholesterol (marker for cholesterol-rich domains) was also observed. In contrast, Shiga toxin (binds endogenous globoside) clustered at the PM but did not co-localize with Prom2 labeled protrusions, suggesting the presence of different kinds of PM domains. Experiments are in progress to examine the distribution of actin relative to these markers. (ii) Depletion of cellular cholesterol which inhibits the formation of PM domains enriched in Bodipy-LacCer, Bodipy-GM1, and cholera toxin, dramatically reduced the length of the Prom2 positive protrusions. We also noted two unexpected effects of Prom2 over-expression. First, membrane
protrusions were more numerous and longer in cells over-expressing Prom2 than in non-transfected cells as judged by fluorescence microscopy of living cells and SEM of fixed cells. Second, Prom2 over-expression caused a dramatic redistribution of caveolin-1 away from the PM. Conclusions: Prom2-positive protrusions are selectively labeled by Bodipy-SL analogs and other lipid raft markers. In addition, our data suggests that Prom2 competes with caveolin-1 for PM cholesterol. Supported by an AHA fellowship to LS and USPHS Grant GM22942 to REP.

2235/B612
Imaging of Mobile Stable Lipid Rafts in the Live Cell Plasma Membrane.
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The organization of the cellular plasma membrane at a nanoscopic length scale is believed to affect the association of distinct sets of membrane proteins for the regulation of multiple signaling pathways. Based on In Vitro results, conflicting models have been proposed which postulate the existence of stable or highly dynamic platforms of membrane lipids and proteins; commonly, these structures are termed membrane rafts. The lack of experimental evidence confirming the existence of putative rafts in living cells has yielded increasing skepticism, casting doubt on a major portion of the recent literature. Here we directly imaged and further characterized lipid rafts in the plasma membrane of living CHO cells by single molecule TIRF microscopy. Using a novel recording scheme for “Thinning Out Clusters while Conserving Stoichiometry of Labeling”¹, molecular homo-association of GPI-anchored mGFP was detected at 37°C and ascribed to specific enrichment in lipid platforms. The mobile mGFP-GPI homo-associates were found to be stable on a seconds timescale and dissolved after cholesterol depletion using methyl-β-cyclodextrin or cholesterol-oxidase. Having confirmed the association of mGFP-GPI to stable membrane rafts, we attempted to use an externally applied marker to test this hypothesis. We used Bodipy-GM1, a probe that was recently reported to be enriched in the liquid-ordered phase of plasma membrane vesicles. When applied to CHO cells at different surface staining, we found that also Bodipy-GM1 homo-associated in a cholesterol-dependent manner, thus providing further evidence for the existence of membrane rafts. To test whether the homo-association is cell-type specific we stained Jurkat T-cells with this marker resulting in similar results. Additional measurements at 25°C showed a much higher loading of plasma membrane rafts with Bodipy-GM1 and point out a model where pre-existing platforms are randomly loaded with this external marker. To show functional relevance of the observed structured we could relate the expression of small heat shock proteins to a disruption of mGFP-GPI homo-associates upon mild heat-shock conditions. 1. Moertelmaier et al., Appl Phys Lett 87, 263903 (2005).

2236/B613
Micropatterning of Plasma Membrane Proteins to Analyze Raft Localization in Living Cells.
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We have developed an assay for quantitative analysis of the interaction between a fluorescently marked protein (prey) and a membrane protein (bait) using microstructured surfaces covered with biotinylated ligands (antibodies) targeted against the bait. The proof-of-concept was demonstrated for the interaction between CD4, a major co-receptor in T-cell signalling, and Lck, a protein tyrosine kinase essential for early T cell signalling. Here we present improvements and a more precise characterization of the method as well as the applicability of the assay for the analysis of protein interactions within lipid rafts in the inner and outer leaflet of the plasma membrane. We stably expressed fluorescently labelled raft and non-raft proteins in the human T24 cell line as prey proteins and determined the degree of interaction with the antibody-targeted bait proteins CD59 (GPI-anchored protein, raft marker) and CD71 (Transferrin-receptor, non-raft marker), respectively. We found strong interaction of CD59 with putative raft markers including
various GPI-GFP constructs, the inner-leaflet associated proteins Lck and Flotillin1 and a Pleckstrin-Homology domain fused to GFP. Importantly, we did not find interaction of CD59 with CD71-GFP and other potential non-raft proteins. When CD71 was used as the bait protein we did not find interaction with the putative raft markers. While the detected absence of CD71 from and the presence of CD59 in lipid rafts confirm current knowledge, it is still very unclear if a lipid-raft dependent coupling of proteins and certain especially negatively charged lipids across the plasma-membrane bilayer exists. Thus, our micropatterning assay will be of great interest to address this question. Schwarzenbacher, M., et al., Micropatterning for quantitative analysis of protein-protein interactions in living cells. Nature Methods, 2008. 5(12): p. 1053-60. Brameshuber, M., et al., Reply to "Uncoupling diffusion and binding in FRAP experiments". Nature Methods, 2009. 6(3): p. 183-184.

**2237/B614**
**Compartmentalization of Signal Regulation by Cytoskeleton-Dependent Membrane Rafts.**
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T cell rafts sequester the Src family kinase Lck from the membrane phosphatase CD45, resulting in down regulation of raft-associated pools of Lck activity. We have shown that the actin cytoskeleton is an important factor in maintaining T cell rafts towards regulating Lck. However, the contribution of the cytoskeleton to Lck regulation in the immunological synapse (IS) of activated T cells has not been shown. Using FRET imaging to measure the nanoscopic properties of the IS, we report here an actin-dependent structuring of rafts in the IS, evidenced by a co-clustering of raft-associated donor-acceptor pairs that was 10-fold greater than the co-clustering of non-raft probes. Furthermore, addition of either filipin or latrunculin B (Lat B), which disrupts actin filaments, specifically disrupted co-clustering of the raft probes in the IS. The filipin also caused targeting of CD45 to the IS, and dephosphorylation of the regulatory tyrosine of Lck. Interestingly, pre-treating conjugates with jasplakinolide, which stabilizes actin filaments, maintained co-clustering of the raft probes in the IS when filipin was added. Jasplakinolide also maintained exclusion of CD45 and Lck regulation in the IS following addition of filipin, and experiments measuring Laurdan fluorescence showed that the jasplakinolide blocked filipin-dependent changes in the lipid environment in the IS. These data show that the actin cytoskeleton maintains a membrane raft environment in the IS that promotes Lck regulation by excluding CD45.co-clustering of raft-associated probes in the immunological synapse (IS) of stimulated T cells resulted in targeting of CD45 to the IS and deregulation of Lck. These data define a role for the actin cytoskeleton in promoting co-clustering of raft-associated proteins, and show this property is important towards regulating raft-associated signaling proteins such as Lck. In stimulated T cells, the actin cytoskeleton maintains a membrane raft environment in the IS that promotes Lck regulation by excluding CD45.

**2238/B615**
**The MAL Protein Controls the Targeting of Specific Molecules to the Immunological Synapse.**
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T cells polarize forming a surface subdomain at the interface with an antigen-presenting cell, known as the immunological synapse (IS). Membrane receptors and signaling molecules assemble at the is into a well-defined structure known as the supramolecular activation cluster (SMAC) which is organized into two differentiated concentric rings, the central SMAC (cSMAC) and the peripheral SMAC (pSMAC). MAL is a highly hydrophobic integral membrane protein consisting of four hydrophobic segments separated by short hydrophilic loops. A peculiar feature of MAL is its selective partitioning into detergent-resistant membrane fractions, which are
believed to be enriched in membrane rafts, of both epithelial MDCK cells and Jurkat T cells. Previous work showed that MAL is an essential element of the machinery for targeting of the influenza virus hemagglutinin (HA) to the apical surface of MDCK cells (1) and that of Lck to the plasma membrane of Jurkat cells (2) by a pathway apparently dependent on the inclusion of these molecules in membrane rafts. The objective of this work was to investigate the distribution and possible role of MAL in T cells forming an is by using confocal microscopy and time-lapse videomicroscopy. We observed that a pool MAL rapidly redistributes to the cSMAC after T cell-APC conjugation. Disruption of the last hydrophilic loop of MAL results in the missorting of the modified MAL molecule to the pSMAC. The ganglioside GM1, a typical raft marker, paralleled the missorting of MAL. Furthermore, the presence of MAL at the pSMAC alters the assembly of the SMAC in such a manner that Lck and LAT, two raft-associated proteins which are normally distributed in the cSMAC, were mistargeted to the pSMAC. Consistent with a role of MAL on the correct targeting of specific raft-associated molecules to the SMAC, ectopically-expressed HA was targeted to either the cSMAC or the pSMAC depending on the expression of intact or modified MAL, respectively. Our results indicate, therefore, that MAL has a role on protein targeting to specific SMAC domains. 1.Puertollano et al. (1999) J. Cell Biol. 145, 141-151. 2.Anton et al. (2008) J. Exp. Med. 205, 3201-3213.

The MARVEL Motif of Occludin Contains the Targeting Information to the Apical Membrane Domain and Tight Junctions in Epithelia.

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MARVEL motifs are M-shaped four transmembrane segments associated with apposition to cholesterol rich domains. Our studies of the human MARVEL motif protein MAL advanced the premise that this motif has two main functions: promote homo-oligomerization and attract liquid ordered phase lipids. These functions are associated with partitioning into- or assembly or stabilization of membrane domains, a process that culminates in targeting of the MARVEL-motif-containing proteins to distinct membrane domains. Tight junctions (TJs) are complex proteinaceous integral membrane assemblies essential for epithelial function. Occludin (Occ), a MARVEL motif containing protein is found in all TJs although its function is unknown. The role of its MARVEL motif has never been addressed as well. We propose that Occ, a key hub protein of the tight junction, interacts with- and affects the proximal lipid environment of TJs via MARVEL-lipid interactions. These lipid-protein interactions are possibly essential for our understanding of the role of Occ in modulating TJ functions. Bi-molecular fluorescent complementation analysis (BiFC) was applied to establish that Occ forms oligomers as it traverses through secretory organelles. BiFC of MAL and Occ demonstrated that these two proteins partition to the same membrane sub-domains. A fluorescent-tagged Occ MARVEL motif containing amino acids 60-269 was generated by deleting the cytosolic C and N terminal segments (FP-MARVELOCC). Live cell confocal microscopy shows that this construct efficiently arrives at the PM in non-polarized cells and is correctly sorted to the apical PM and TJs in polarized MDCK cells. Site directed mutagenesis is currently applied to determine the role of the transmembrane segments’ length as well as intra-membrane aromatic amino acids in MARVELOCC function. These data demonstrate that the MARVEL motif is involved in Occ oligomerization and in the targeting of Occ to TJs possibly via partitioning into or stabilization of liquid ordered domains proximal to TJs.

Sphingomyelin Undergoes Dynamic Downregulation in C2C12 Myoblasts and Intracellular Location Coincides with Proliferation and Differentiation Activities.

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Membrane rafts are sphingomyelin (SM) and cholesterol enriched membrane platforms that regulate skeletal muscle differentiation. SM, a source of ceramide and sphingosine-1-phosphate (S1P) lipid messengers, mediates myoblast growth and differentiation when SM is expressed
intracellularly. However, SM signaling is poorly understood. C2C12 myoblasts and lysenin toxin binding by immunofluorescence were used to determine SM content. Myoblast SM levels were assessed under continuous neutral β-sphingomyelinase (SMase) and methyl-β-cyclodextrin (MBC) treatments post differentiation induction. SM is present in one of three states: 1) covering the whole cell; 2) intracellular punctuate and around nucleus; or 3) absent from the cell. Myoblasts plated in proliferation medium for 5 hours all were covered whole with SM and persisted for 24 hours. By 48 hours and just before differentiation stimulation, SM levels decreased 3-fold and more cells expressed punctuate SM. By 72 hours SM declined further and was highly punctate or absent in ~75% of myoblasts although afterwards SM levels were stable to 120 hours. Likewise, differentiating myoblasts exposed to SMase or MBC rapidly declined in SM over 72 hours with ~85% of myoblasts being punctuate or absent in SM. The SM content was always lower than controls by about 29% starting at 24 hours in differentiation. Furthermore, SMase treated myoblasts were analyzed for effects on differentiation by myosin heavy chain (MHC) expression and were found to be delayed for muscle formation for 96 hours versus controls that expressed MHC between 48 and 72 hours. Additionally, a re-emergence of SM over whole myoblasts in some cases was found by 96 hours to correlate with MHC expression. We conclude that SM location is important to signaling of myoblast proliferation or differentiation with internalized SM correlating with proliferation and plasma membrane intracellular SM associated with differentiation. NSF-IOS-0821324; NIH-NCMHHD-P20MD000544.

2241/B618
***ABO Blood Group Glycans Modulate Sialic Acid Recognition on Erythrocytes.***
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**Background/Objectives:** ABH(O) blood group polymorphisms are intra-species variations in blood cell surface glycan structures in humans and other primates. While pathogen interactions are suggested as the evolutionary driving force and some associations with bacterial and viral infections are described, direct binding of lethal pathogens to ABH antigens has not been reported. We hypothesized that the spatial organization of other cell surface glycans might be non-covalently modulated by ABO blood group glycans, thus affecting key processes such as recognition of sialic acid-containing glycans. **Results:** We found that ABH antigens on human erythrocytes (RBCs) differentially modulate interactions of three sialic-acid recognizing proteins: human Siglec-2 (CD22), the pandemic human influenza hemagglutinin (1918-South Carolina) and the plant lectin *Sambucus nigra* agglutinin (SNA). While all three proteins have the same binding preference In Vitro (recognizing Sias in α2-6-linkage) and none can directly bind ABH antigens, each interacts with RBCs in a unique pattern. Siglec-2 and SNA bind in distinct clusters on erythrocyte membranes that are stabilized by a and B antigens. Using specific glycosidases that convert a or B glycans to the underlying H(O) structure, we confirm that they stabilize sialylated glycan clusters uniquely for each blood type. We propose a model for spatial organization of sialylated glycan clusters on RBC surfaces, which generates differential interactions with erythrocytes from each blood type despite having a similar sialic acid content. Indeed, the binding preference of the invasive merozoite erythrocyte-binding antigen (EBA)-175 of *Plasmodium falciparum* (the major cause of malaria mortality) is also affected by ABH antigens, influencing its primary specificity for recognizing α2-3-linked sialic acids on glycoporphin A. **Conclusion:** By non-covalently stabilizing and modifying glycan clusters, ABH blood group antigens modulate interactions involving sialic acids without being direct ligand themselves. As sialic acids play important roles in many physiological and pathological interactions, this novel finding provides an alternate explanation for the evolutionary persistence of ABO polymorphism.

2242/B619
***The Role of Sterol-Rich Membranes in Growth Site Positioning in Fission Yeast.***
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Polarization in fission yeast involves cytoskeleton-mediated positioning of growth sites. Actin is believed to localize and confine the growth machinery, while microtubules regulate growth site positioning by depositing marker proteins at the cell poles. These are thought to affect actin organization by recruiting the formin for3p. We introduce a new element in this picture: sterol-rich membranes, and study their role in the process of positioning of the growth sites. Using cells recovering from starvation we image de novo polarity establishment. Automated image analysis software was developed to analyze the dynamics of sterol-rich membranes and the dynamics of cell growth. We show that sterol-rich membranes, and with them the growth machinery, are initially unpolarized and only polarize with growth initiation. We also show that tea1p controls polarized growth by positioning sterol-rich membranes. Sterol-rich membrane positioning thus represents a crucial missing link between cytoskeleton function and cell polarization.

2243/B620

Cell Wall Stress Affects the Nuclear Diffusion Barrier in Budding Yeast.
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We recently showed that a diffusion barrier in the nuclear membrane of the budding yeast Saccharomyces cerevisiae is important to confine aging factors such as extrachromosomal rDNA circles in the mother cell during cell division (Z. Shcheprova, et al, Nature 454, 728). Interestingly, mutants with a defective diffusion barrier are long-lived and have an impaired rejuvenation of daughter cells. It is established that many mild stresses can induce longevity in yeast cells. We wondered whether life span extension could also in these cases be due to barrier breakdown and redistribution of aging factors between mother and bud. To investigate how different stresses influence diffusion barriers in the nuclear envelope and in the ER, we employed fluorescence loss in photobleaching to measure the diffusion of membrane bound proteins across the bud neck in mitotic cells. We find that cell wall stress decreases barrier strength in the nuclear envelope bud not in the ER. This inhibition of the barrier depends on Rho1p and the Pkc1p kinase but not on the downstream MAP kinase pathway. Since mutant cells that have not a polarized actin cytoskeleton such as bud6Δ and bni1Δ cells have also an impaired diffusion barrier, we speculate that the cell wall stress response may act on the diffusion barrier via the actin cytoskeleton. It will be interesting to determine whether stress induced longevity may be achieved by the regulation of diffusion barriers that control the segregation of potential senescence factors in mitosis.

2244/B621

Role of the Lamin-Binding-Protein Homolog Src1 in the Nuclear Architecture of Budding Yeast.
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During vegetative growth, Saccharomyces cerevisiae cells divide asymmetrically through budding from a larger mother cell to produce smaller daughter cells. In the past few years, our lab has described diffusion barriers in the plasma membrane and the endoplasmic reticulum (ER) that define specific compartments within the dividing yeast cell. Recently, we also showed that during the closed mitosis of budding yeast, a diffusion barrier located in the envelope of anaphase nuclei contributes to the rejuvenation of daughter cells. Because membrane organization is important for asymmetric cell division, we further investigated the genetic requirements of nuclear membrane compartmentalization in yeast. To identify novel genes involved in the compartmentalization of the outer nuclear membrane (ONM) during mitosis, we used a fluorescence loss in photobleaching (FLIP) assay to search for mutations that abolish the separation of Nsg1-GFP, an ONM protein, into mother and daughter specific pools. Through this screen, we identified Src1 and Heh2 as being required for the compartmentalization of specific ONM proteins. Src1 and Heh2 are the yeast homologs of the mammalian lamin-binding proteins MAN1 and LEM2 and reside in the inner nuclear membrane (INM). Our current data indicate that the loss of nuclear diffusion barrier observed in src1Δ cells is caused by a general disorganization of the ER, specifically a lack of separation between the ONM and the ER, two membrane systems that are physically continuous with each other yet show distinct protein compositions. Furthermore the two
are organized differently: The ER consists of tubules and sheets while the ONM forms one sheet on the surface of the INM leaving holes for the nuclear pore complexes. We propose that the INM proteins Src1 and Heh2 play a major role in differentiating the ONM from the rest of the ER.

2245/B622
**Vesicular or Non-Vesicular Transport? Osh4p, a Putative Sterol-Transfer Protein, Is a Direct Regulator of Vesicle Docking during Polarized Exocytosis.**

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Oxysterol-binding protein (OSBP)-related protein Kes1/Osh4p is implicated in nonvesicular sterol transfer between membranes within yeast cells. To test whether Osh4p or the six other yeast Osh proteins are sterol "lipid transfer proteins" ("LTPs"), we studied endoplasmic reticulum (ER)-to-plasma membrane (PM) sterol transport in OSH deletion mutants lacking one, several, or all Osh proteins. In a mutant conditional for OSH function, ER-PM ergosterol transport slowed ~20-fold compared with cells expressing a full complement of Osh proteins. Although this initial finding suggested that Osh proteins act as sterol LTPs, the situation is far more complex. Analysis of transport rates in cells expressing all Osh proteins but Osh4p, or cells expressing just Osh4p alone, indicated that Osh4p itself does not function as a nonvesicular sterol LTP. Instead, we found that Osh4p tracked on post-Golgi vesicles that move from mother cells into budding daughter cells. Within buds, Osh4p transited to sites of polarized growth on the PM, and undocked vesicles accumulated within buds in the absence of Osh proteins. Consistent with our genetic results, Osh4p formed complexes In Vivo with the small GTPases Cdc42p, Rho1p, and Sec4p, and the exocyst protein Sec6p. Osh proteins are required for a specific pathway of polarized exocytosis, suggesting that this is how Osh proteins affect Cdc42p- and Rho1p-dependent polarization. Although Osh4p had a direct effect on polarized exocytosis, the role of Osh4p in sterol trafficking was unclear. Contrary to what is predicted for a sterol transporter, the inability of a specific Osh4 mutant protein to bind sterols did not cause its inactivation, but rather caused its hyperactivation and dominant lethality. These results suggested that Osh4p has an independent role in sterol trafficking but not as a transporter. Using sterols and other lipids as spatial landmarks, we propose that Osh4p recognizes when vesicles are closely apposed to the PM and only then permits vesicle docking to occur at polarized sites.

2246/B623
**TAT-5 Regulates Cell Polarity and Endocytosis.**

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Contact between cells can induce inner-outer (apical-basal) polarities that are vital for many processes, including cell movement and epithelial function. The PAR proteins are known regulators of polarity. at the 4 cell stage in *C. elegans*, the anterior PAR proteins PAR-3, PAR-6, and PKC-3 are initially localized symmetrically around the plasma membrane. within one cell cycle, they are relocalized away from cell contacts and enriched on the outer surface of cells. How these proteins are moved away from cell contacts is not known. Previous work has placed the anterior PAR proteins in control of endocytosis (Balklava *et al.*, 2007). In an RNAi screen for regulators of cell contact-mediated PAR polarity, we identified several genes implicated in endocytosis and membrane trafficking. Polarized trafficking is a promising mechanism for transporting the PAR proteins away from cell contacts. One interesting protein identified in the screen is TAT-5, a putative aminophospholipid translocase. RNAi of tat-5 results in multiple endocytic defects similar to anterior par mutants, such as in recycling endosomes and clathrin-mediated endocytosis. In addition, tat-5 RNAi embryos have a novel defect in the endocytosis of midbodies after abscission. We have analyzed several previously identified endocytic mutants and have been unable to recapitulate the polarity defect of tat-5 mutants. Loss of TAT-5 also
disrupts the structure of cell contacts by the introduction of tubulovesicular structures between cells. Establishing the cell biological mechanism for relocalization of the PAR polarity proteins may inform the mechanism behind other cell contact-induced polarization events, such as inner-outer polarization during compaction of the mammalian embryo and apicobasal polarization of epithelial cells.

Endocytosis (2247 – 2268)

2247/B624
The Role of Cargo Valency in Clathrin-Mediated Endocytosis.
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Clathrin-mediated endocytosis (CME) in mammalian cells is critical for many cellular processes including cell surface receptor down-regulation and nutrient uptake. Endocytosis of constitutively internalized transferrin receptors (TfnR) requires the recognition of a sorting motif in the cytoplasmic tail by the adaptor protein AP-2. We and others have shown that efficient endocytosis of TfnR is saturable, and that over-expression of TfnRs does not change the density of clathrin-coated pits (CCPs). These observations suggest that other factors, which might be titrated by over-expressed receptors, are limiting CME. On this basis, we propose to test whether acute local increases in TfnR concentration by clustering could initiate de novo CCP formation and affect the kinetics of receptor internalization. To this end, we engineered site-specific biotinylation of TfnR and applied purified chimeric streptavidin bearing 0, 1, 2, 3, or 4 biotin binding sites. Interestingly, we found that cell surface CCP density increases with streptavidin valency. Furthermore, we used live cell dual-color total internal reflection fluorescence microscopy and computational analysis to examine the kinetic differences of CME in response to different streptavidin valency. Our results suggest that a local increase in cargo concentration by clustering may affect CME efficiency, possibly by potentiating the interaction between AP-2 and the sorting motif of TfnR.

2248/B625
Clathrin Adaptors Epsin 1 and AP-2 Differentially Regulate Signaling and Trafficking of Protease-activated Receptor-1.
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Protease-activated receptor-1 (PAR1), a G protein coupled receptor (GPCR) for thrombin, is irreversibly proteolytically activated. Hence, PAR1 signaling is tightly regulated. PAR1 signaling is regulated predominantly by phosphorylation-dependent desensitization and clathrin-mediated endocytic trafficking, which occurs independent of arrestins. Unactivated PAR1 is slowly constitutively internalized, whereas activated PAR1 is rapidly internalized through a phosphorylation-dependent mechanism. We previously showed that PAR1 constitutive internalization requires adaptor protein complex-2 (AP-2), a C-tail tyrosine based-motif YKKL and de-ubiquitination of the intervening lysine residues. Thus, ubiquitination negatively regulates PAR1 constitutive internalization. In contrast, ubiquitination of activated PAR1 specifies a distinct clathrin adaptor requirement for internalization that is independent of AP-2 and arrestins. The broadly expressed epsins bind ubiquinated cargo and facilitate internalization, suggesting a function in regulation of ubiquitinated GPCR signaling and trafficking. To assess epsin and AP-2 function, we examined PAR1 signaling and trafficking in cells depleted of epsin and/or AP-2 by siRNA. Here, we demonstrate that both epsin and AP-2 mediate activated PAR1 internalization. However, only epsin required ubiquitination of PAR1 for ligand-induced internalization. Interestingly, activated PAR1 signaling was also enhanced in epsin depleted cells expressing wildtype and ubiquitination-deficient PAR1 without affecting initial coupling to effector signaling, suggesting that epsin is involved in PAR1 signal termination. Remarkably, activated PAR1 signaling was significantly increased in cells depleted AP-2. However, initial signaling by a PAR1 mutant defective in phosphorylation and internalization was unaffected. Thus, AP-2 appears to
function in PAR1 uncoupling from effector signaling though a process that requires phosphorylation and internalization but not receptor ubiquitination. Together these findings suggest that different clathrin adaptors differentially regulate GPCR desensitization and internalization through novel mechanisms that require distinct receptor posttranslational modifications.

2249/B626  
**Correlative Intensity Timecourse Analysis in Endocytosis.**  
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High-resolution imaging can resolve both spatial and temporal dynamics of clathrin-coated pit (CCP) maturation, and the recent development of automated detection and tracking methods has allowed us to measure all CCPs visible in total internal reflection fluorescence microscopy (TIR-FM) in BSC1 cells (Jaqaman et al., 2008, Loerke, Mettlen et al. 2009). Fluorescence intensity timecourses of clathrin and other coat proteins contain valuable mechanistic and temporal information about coat maturation. However, intensity analysis is far from trivial: While averaging of noisy intensity signals is desirable to visualize trends in low-intensity objects, excessive averaging can obscure informative heterogeneities in CCP behavior. In addition, intensity fluctuations in TIR-FM incorporate both fluctuations in objects’ size and their distance from the membrane. We have developed an intensity analysis assay that retains heterogeneity between different lifetime CCP cohorts, and preserves fast dynamics within the cohort. In multi-channel experiments visualizing EGFP and mCherry-tagged endocytic factors, it allows us to directly observe the association of coat proteins across the entire CCP lifetime. The distinct contributions of protein recruitment vs. spatial redistribution to the measured intensity changes can be deconvolved using dual-channel EPI/TIRF illumination. We observe distinct phases in clathrin maturation, which we term initiation, stabilization/plateau and departure phase, and CCPs of different lifetimes differ primarily in the length of the stabilization/plateau phase. The slope of clathrin intensity during the initiation phase is constant, whereas the slope of the adaptor protein AP2 intensity increases as a function of lifetime up to ~30s, suggesting that in newly initiated pits, continued maturation depends on their ability to recruit AP2. During the maturation of productive CCPs, we observe the divergence of the intensity signals between clathrin and AP2, and between TIRF and EPI. Our findings indicate that AP2 is a key factor in satisfying the endocytic checkpoint requirement during stabilization, that it undergoes spatial redistribution within CCPs, and dissociates from pits prior to CCP internalization.

2250/B627  
**Modeling the Bioenergetics of Protein-Mediated Vesiculation in Clathrin-Dependent Endocytosis.**  
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Internalization of extracellular cargo via the clathrin-dependent endocytosis (CDE) is an important regulatory process prominent in several cellular functions. Subsequent to receptor activation, a sequence of molecular events in CDE is responsible for the recruitment of various accessory proteins such as AP-2, epsin, AP180, eps15, dynamin, amphiphysin, endophilin and clathrin to the plasma membrane to orchestrate membrane vesiculation. While the involvement of these proteins have been established and their roles in membrane deformation, cargo recognition, and vesicle scission have been identified, current conceptual understanding falls short of a mechanistic description of the cooperativity. Using a computational framework, we describe the energetics of deformations membranes by using the Helfrich Hamiltonian represented in a curvilinear coordinate system. In our model, curvature inducing proteins and protein assembly like epsin and clathrin coat affect the membrane Hamiltonian by changing the preferred mean curvature of the membrane. We argue that the energy required to deform the membrane from a
planar state to a curved state can be offset by stabilizing interactions between the proteins in the clathrin coat assembly and between the coat proteins and the membrane. Using the experimentally obtained values of protein-protein and protein-membrane interactions in clathrin coat, we demonstrate the energetic stabilization of a budding vesicle induced by the clathrin-coat assembly. For a 50 nm diameter vesicle, we establish that about 22 epsins are present per matured vesicle. Our model predictions are in good agreement with the experimental observations that inhibiting epsin-clathrin interaction arrests the clathrin-coated vesicles to the early phase of vesicle growth. Furthermore, according to the predictions of our model, disrupting the epsin-membrane interaction should make the coated vesicular bud highly unfavorable. Indeed, in cells microinjected with ENTH antibodies the extent of clathrin-coated structures decreases remarkably. In conclusion, our bioenergetic model quantitatively explains several experimental observations on the process of vesicle nucleation induced by the clathrin-coated assembly.

2251/B628
The Transmitter-Release Site Associated Endocytosis Complex Is Not Tethered By F-Actin.
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Synaptic vesicles (SVs) are triggered to discharge their contents at specialized transmitter release sites (TRS) by calcium ion influx through nearby tethered calcium channels within the active zone (AZ) region of the presynaptic terminal. These SVs are then recaptured by endocytosis at specialized sites in the peri-AZ region. SV endocytic proteins have been localized to this area by both light and electron microscopy. Our recent report (Khanna et al., Eur J Neurosci. 26:560, 2007) provides biochemical evidence for a direct molecular link between the TRS core and an 'endocytosis module' that differs in protein composition from a more remote, 'classical' endocytosis apparatus. These observations support the hypothesis that the TRS core is linked by a cytoplasmic matrix scaffold to a peri-endocytosis module. Objective: Since f-actin has been localized to the peri-AZ region by immunogold microscopy, we have used the actin disrupting agent, latrunculin A, to test the hypothesis that f-actin links the TRS- and endocytosis compartments. Methods: High resolution imaging and immunocytochemistry of dissociated chick ciliary ganglia (CCG) synapses with the N-type Ca2+ channel (CaV2.2) and H-clathrin were used to mark the TRS core and endocytosis apparatus, respectively. Intensity Correlation Analysis (ICA), a test of whether two protein stains covary, was used for quantitative analysis. Results: Clathrin-stained patches were found to abut Cav2.2 clusters at the release face in the presynaptic terminal and a distinct endocytic complex from the TRS complex was identified by Fractional Recovery analysis. Latrunculin A treatment was observed to reduce f-actin staining but Cav2.2 cluster remain associated with clathrin stained patches. Conclusion: We find that loss of f-actin did not dissociate the exo- and endocytosis complexes and, hence, that these entities must be linked by some other bridging component.

2252/B629
Clathrin-mediated Endocytosis Is Organized Into Hot Spots by the Actin Cortex.
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Clathrin-mediated endocytosis (CME) is the main mechanism by which cells internalize molecules from the extracellular space. As such, it is involved in all functions regulating cellular homeostasis. While some work has suggested that CME tends to reoccur at 'hot spots', sites of preferred nucleation of clathrin coated pits (CCPs), other data has suggested that these same sites are the result of random CCP nucleations restricted spatially by areas of no nucleation on the cell membrane (Gaidarov et al.,1999; Ehrlich et al., 2004). The goal of this research is to
resolve whether CME is spatially organized by hot spots, and to identify the components responsible for this putative organization. Using Total Internal Reflection Fluorescent Microscopy (TIRFM) in conjunction with computational image analysis of the spatial dynamics of CCPs, we show that ~60% of CCPs nucleate at hot spots of ~200 nm in radius rather than being randomly distributed. We found that depolymerization of the actin cortex with LatrunculinA increased the number of nucleations overall, but increased the relative amount of nucleations in hot spots. This result suggests that hot spots may be defined by particularly stable patches of the actin cortex where endocytic nucleation factors accumulate. Consistent with this hypothesis partial depletion PIP(4,5)2 and adaptor protein AP2, displayed the same phenotype as actin depolymerization. We screened 15 partial depletions of various endocytic proteins and found that reduction of Snx9 caused a strong disruption of the organization of CME. We tracked the recruitment of Snx9 to assembling pits by dual-color TIRFM and found that inside hot spots Snx9 is present before the onset of clathrin assembly, whereas outside hot spots it begins to accumulate simultaneously with clathrin. This data suggests that Snx9 is more stably associated with hot spots where, as a scaffolding protein capable of binding diverse members of the endocytic machinery, it may facilitate the local association of the components necessary for CCP nucleation. We conclude that hot spots are defined as individual actin cortex mesh sections that contain and more stably retain the endocytic proteins necessary to preferentially nucleate CCPs.

2253/B630
Characterization of Two Novel Binding Partners for the Lowe Syndrome Protein OCRL1.
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Lowe syndrome is an X-linked developmental disease that causes defects in the kidney, brain and eyes. The disease is caused by mutations in a ubiquitously expressed inositol polyphosphate-5-phosphatase, called OCRL1. It is currently unclear how the loss of this enzyme causes the disease. In an effort to identify novel areas of OCRL1 function we have identified two novel related binding partners to OCRL1 (and a closely related protein called Inpp5b). Both proteins contain an N-terminal PH domain and bind to the OCRL1 ASH/Rho GAP-like domain via a conserved motif in their C-terminus. Disease relevant mutations of OCRL1 cause loss of binding. Both proteins have a nuclear and trans-Golgi localisation and under certain conditions are visible on endosomes. They share a common OCRL1 binding site with the endocytic signalling adaptor protein APPL1 and our results suggest they play a role in cellular signalling events. Further characterization of these proteins may give insights into how loss of OCRL1 causes Lowe syndrome.

2254/B631
High Resolution 3D Single Molecule Tracking with Multifocal Plane Microscopy Reveals Complex Endocytic and Exocytic Pathways In Live Cells.
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The study of intracellular events on the endocytic/exocytic pathway is of fundamental importance to understanding cellular function. Past research efforts have mainly focused on studying these events either on the plasma membrane or at the cell interior. However, the 3D trafficking itineraries that span from the plasma membrane to the intracellular compartments have not been well studied. The main reason for this is that current microscopes are not well adapted for imaging rapidly moving intracellular objects in 3D. Recently we developed a microscopy modality, multifocal plane microscopy (MUM), in which several planes can be simultaneously imaged within the cell [1]. This has allowed us to quantitatively track and characterize 3D intracellular events that are otherwise difficult to capture with conventional imaging techniques [1-3]. Our data shows evidence for a direct delivery pathway where a tubule extends from a sorting endosome to an exocytic site on the plasma membrane [2]. In some exocytic events, we observed a 'triggering' mechanism where a collision of a small vesicle preceded the fusion event [2]. Using quantum dot

2255/B632
Role of Key Conserved Residues of the VPS9 of RAP6 Domain on the Function of Rab5.
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Rab5 is a key small GTPase involved in several steps during the uptake of ligands (i.e., EGF, Insulin and Transferrin). Specifically, Rab5 plays a key role in the early stages of internalization, including endosome fusion. Several intracellular molecules, including Rabex-5, Ras Interference 1 (Rin1), Alsin, and Rab5 Activating Protein (RAP) 6, have been shown to play a key role during receptor-mediated endocytosis by modulating the activity of Rab5. In this study, we examined the role of key conserved amino acids in RAP6 on the activation of Rab5 by preparing seven point mutants in the Vps9 domain of RAP6. Initial experiments indicate that Rin1: D1381A, Rin1: P1385A, Rin1: Y1405F and Rin1: Y1420F poorly interacted with Rab5 as compared with Rab5: wild type. However, Rin1: Y1348F, Rin1: E1417A, and Rin1: Y1410F mutants were able to interact with Rab5. We also found that the expression of RAP6: wild type induced the formation of enlarged Rab5-positive endosomes and more importantly, RAP6 partially co-localized with Rab5: wild type. Furthermore, two of them (Rin1: D1381A and Rin1: Y1420F) showed a diffuse cellular distribution. Taken together, these data suggest key amino acids in the Vps9 domain of RAP6 play an important role on the regulation of Rab5 function.

2256/B633
MARCH Proteins Promote Delivery of Clathrin-Independent Endocytic Cargo Proteins MHCI and CD98 to Late Endosomes.
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Our lab has focused on studying the internalization and trafficking of plasma membrane proteins that enter cells through Clathrin-Independent Endocytosis (CIE), including major histocompatibility complex Class I protein (MHCI). In recently published work, we used a proteomic-based screen of early CIE endosomes to identify new cargo proteins trafficking in the CIE pathway. Some identified cargo proteins follow a similar itinerary to MHCI, merging with components of the Clathrin-Dependent Endocytic (CDE) pathway in EEA1 positive early endosomes. MHCI subsequently traffics to late endosomes (LE) for degradation or recycles to the cell surface in CIE recycling tubes. Interestingly, a subset of new cargo proteins, typified by CD98, do not traffic to EEA1 endosomes but are seen prominently in CIE recycling tubes. CD98 is a multifunctional protein, comprising the heavy chain of neutral amino acid transporters and interacting with integrins to promote cell adhesion. Here we investigated in more detail the itinerary and fate of CIE cargo proteins. We found that some cargo proteins, including MHCI, that reach the EEA1 early endosomes accumulate after 24h in LE if degradation is inhibited. However, cargo proteins that do not reach the EEA1 compartment, like CD98, are largely absent from LE after 24h. Work from many labs has demonstrated that the viral proteins K3 and K5 can down regulate cell surface levels of MHCI in virally infected cells. K3 and K5 are membrane associated E3 ubiquitin ligases. Ten membrane-associated RING-CH (MARCH) proteins have been identified as human homologues of K3 and K5. Over-expression of MARCH4 in HeLa cells
causes loss of MHCI from the cell surface likely through ubiquitination of the MHCI cytoplasmic tail. We found that over-expression of MARCH4 enhanced delivery of internalized MHCI to LE and resulted in the loss of MHCI from recycling tubules. Interestingly, over-expression of MARCH1, but not MARCH4, led to a profound change in CD98 trafficking, causing it to now accumulate in LE over several hours. Therefore, MARCH proteins may play an important role in specifying CIE cargos for degradation, possibly through ubiquitination of their cytoplasmic tails.

2257/B634
Rabex-5: A Novel Rab22 Effector That Mediates a Rab22-Rab5 Signaling Cascade in Endocytosis.
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Rabex-5 targets to early endosomes and functions as a guanine nucleotide exchange factor (GEF) for Rab5. Membrane targeting is critical for Rabex-5 to activate Rab5 on early endosomes in the cell. Here we report the identification of Rab22 as a binding site on early endosomes for direct recruitment of Rabex-5 and activation of Rab5, establishing a Rab22-Rab5 signaling relay to promote early endosome fusion. Rab22 in GTPγS-loaded form, but not GDP-loaded form, binds to the EET domain (residues 81-230) of Rabex-5 in pull-down assays. Rabex-5 targets to Rab22-containing early endosomes and Rab22 knock-down by shRNA abrogates the membrane targeting of Rabex-5 in the cell. In addition, co-expression of Rab22 and Rab5 shows synergistic enlargement of early endosomes, and this synergy is dependent on Rabex-5, providing further support for the collaboration of the two Rab GTPases in regulation of endosomal dynamics. This novel Rab22-Rabex-5-Rab5 cascade is functionally important for the endocytosis and degradation of epidermal growth factor (EGF).

2258/B635
MICAL-L1, a Dynamic Link Controlling EHD1/Rab8a Tubular Recycling Endosomes.
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The cell surface localization of receptors is regulated by the rate of receptor internalization and recycling. Recent studies have identified the C-terminal Eps15 homology domain (EHD) containing protein, EHD1, as a critical regulator of receptor recycling from the endocytic recycling compartment (ERC) to the plasma membrane. EHD1 associates with a series of tubular membranes that emanate from the perinuclear ERC. Our data demonstrates that EHD1 tubules are essential for efficient recycling to the cell surface. However, the mechanism regulating EHD1 association with tubules remains unclear; although EHD proteins can tubulate membranes in vitro, EHD1 primarily associates with pre-existing tubular membranes in vivo. We therefore sought to identify novel interacting partners that associate with EHD1 and regulate its association with tubules. Pull-down experiments using GST-EH domain of EHD1 as bait identified MICAL-L1 (molecule interacting with CasL-like 1), a largely uncharacterized member of MICAL-family of proteins that contains two asp-pro-phe (NPF) motifs, sequences that typically bind to EH domains. We found that MICAL-L1 interacts with the EHD1 EH-domain via its first NPF motif, and co-immunoprecipitation experiments from transfected HeLa cell lysates demonstrated that the interaction also occurs in vivo. A high level of co-localization of both endogenous and overexpressed MICAL-L1 with EHD1 was observed, primarily on the tubules. The MICAL-L1 C-terminal coiled coil was necessary for its tubular membrane association, but surprisingly, the presence of EHD1 was not required. Previous studies reported an interaction between MICAL-L1 and GTP-bound Rab8a, a protein that localizes to EHD1 containing tubules. We found that MICAL-L1 was required both for the interaction between EHD1 and Rab8a and for their association with tubular membranes. Finally, SiRNA-depletion of MICAL-L1 significantly impaired the recycling of transferrin and β1-integrin receptors suggesting that like EHD1, MICAL-L1 also regulates recycling of receptors back to the plasma membrane. These data implicate MICAL-L1
as a critical regulator of endocytic recycling that bridges the interaction between EHD1 and Rab8a on the tubular ERC.

2259/B636

**A Novel Adaptor Protein That Regulates Ubiquitination of the B2 Adrenergic Receptor.**

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The β2 adrenergic receptor (β2AR) is a prototypic G-protein coupled receptor that is internalized and degraded upon agonist stimulation. Using a shRNA (short hairpin RNA) -based screen, we identified an arrestin domain containing protein, TLIMP, as a putative regulator of β2AR. Decreased intracellular levels of TLIMP hindered isoproterenol agonist-promoted ubiquitination and degradation of β2AR. TLIMP interacted efficiently with the β2AR -specific ubiquitin ligase NEDD4. In addition, TLIMP was actively recruited to the receptor following β2AR stimulation. Finally, knockdown of TLIMP completely inhibited the interaction between NEDD4 and β2AR. Our results suggest that TLIMP functions as a specific regulator of β2AR ubiquitination and degradation.

2260/B637

**Distinct Sorting Determinants Guide the Trafficking Itinerary of the New Clathrin-independent Endocytic Cargo Proteins CD44 and CD147.**

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In recent years, it has been demonstrated that different mechanisms of clathrin-independent endocytosis (CIE) mediate the internalization of integral membrane proteins devoid of clathrin- or clathrin-adaptor targeting sequences. In HeLa cells, CIE cargo, such as the major histocompatibility complex Class I protein (MHCI) and the interleukin 2 receptor α-subunit (Tac), enters the cell in non-clathrin vesicles that later fuse with EEA1- and transferrin-positive endosomes. Eventually, the cargo is either degraded in late endosomes or recycled back to the plasma membrane through recycling tubules. Although, in HeLa cells this pathway is the preferred route for many CIE cargoes, our group has identified two new CIE cargo proteins, CD44 and CD147 that differ in their trafficking itinerary from MHCI/Tac once captured in CIE endocytic vesicles. CD44 and CD147 do not travel to EEA1-positive endosomes. Instead, they are routed directly to the recycling tubes, avoiding any encounter with cargo derived from clathrin-dependent endocytosis (CDE). These observations suggest that sorting mechanisms may dictate the processing and final destination of CIE cargo. To identify potential sorting determinants in the sequence of CD44 and CD147, we created chimeras consisting of the luminal domain of Tac and the transmembrane and cytoplasmic domains of CD44 or CD147 (Tac-CD44-CD44 or Tac-CD147-CD147) and examined their itinerary after endocytosis using antibody internalization experiments. In contrast to the trafficking of Tac, the chimeras by-passed EEA1-positive endosomes, evaded late endosomes and accumulated in recycling tubules. The overall distribution and traffic of the chimeras resembled that of CD44 and CD147, indicating that these proteins contain sequence information in the cytoplasmic and/or transmembrane domains that alters the trafficking itinerary of Tac. Currently, we are assessing the role the cytoplasmic and transmembrane domains play in sorting and whether Tac cytoplasmic domain also contains targeting information. Based on these observations, we propose that distinct sorting determinants regulate the itinerary and final destination of different groups of CIE cargo molecules.

2261/B638

**Endocytosis Mediated by ICAM-1 Occurs in Cholesterol-, Sphingomyelin- and Cholera Toxin-Positive Domains but Is Independent of Caveolae.**

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Intercellular adhesion molecule 1 (ICAM-1) is a transmembrane glycoprotein overexpressed by endothelial cells in many pathologies and it serves as an adhesion molecule for leukocytes. Binding of artificial multivalent ligands to ICAM-1, such as anti-ICAM polymer particles in the nanometer or micrometer scale, activates a signal cascade that mediates endocytosis, which can be used for drug delivery to the vascular endothelium. This pathway, called cell adhesion molecule (CAM)-mediated endocytosis, differs from classical endocytosis mediated by clathrin or caveoli, macropinocytosis and phagocytosis. However, both ICAM-1 location in the endothelial plasmalemma and signaling mediated by this molecule have been associated to caveoli. To clarify the potential association of CAM-endocytosis to caveoli and/or lipid rafts, we used 100 nm and 5 µm FITC-labeled latex particles coated with anti-ICAM to investigate their binding location and endocytosis in TNFα-activated endothelial cell cultures and lung endothelial cells in mice, by fluorescence and transmission electron microscopy. Filipin staining and loading of cells with the BODIPY FL C12-sphingomyelin analog showed that anti-ICAM particles bound to the endothelial surface on areas enriched both in cholesterol and sphingomyelin. These regions were positive for ganglioside GM1, as observed by immunostaining with an antibody to this marker. This result was confirmed by localization of anti-ICAM particles to areas enriched in fluorescent cholera toxin B on the cell surface. Removal of cholesterol by methyl-β-cyclodextrin, but not treatment with filipin, inhibited endocytosis of anti-ICAM particles. After IV injection in mice, electron microscopy of the pulmonary vasculature showed anti-ICAM particles being internalized by endothelial cells into endocytic vesicles morphologically different from clathrin and caveolar compartments. Finally, In Vivo endocytosis of anti-ICAM particles was also observed in caveolin-1 knockout mice. These results demonstrate that although ICAM-1 location on the cell surface may be associated to lipid raft-like domains and its signaling may depend on these platforms, ICAM-1-mediated CAM-endocytosis is independent of caveoli. AHA 09BGIA2450014.

2262/B639
A New Feature of the Stress Response: Hsp70-Mediated Increase in Endocytosis.
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The expression of heat shock proteins (hsp) is a well conserved cellular response to stress. These proteins participate in the refolding of denatured polypeptides after an array of insults as well as the stabilizing of critical cellular processes. Recently, several hsp have been shown to associate with membranes, in particular Hsp70, the stress-inducible member of the Hsp70 family. Hsp70 has been detected on the surface of transformed or stressed cells. Although the role of membrane-associated hsp in the stress response is unclear, it is possible that they participate in membrane-associated processes, such as cell adhesion, transport, receptors expression, and internalization of macromolecules. We report that endocytosis of transferrin, which is involved in the delivery of iron to the cell, was increased after heat shock or after incubation with inhibitors of Hsp90 function. In both cases, the increase in endocytosis was reverted by inhibition of transcription, suggesting that gene expression is required. Transfection of cells with the Hsp70 gene or inhibition of its expression by RNAi confirmed the role of this hsp in the increase of endocytosis. The mechanism for the enhancement of transferrin uptake was related to an accelerated internalization of the ligand-receptor complex as well as an increase in receptor recycling. These observations constitute a new paradigm for the cellular protection induced by hsp.

2263/B640 ABSTRACT WITHDRAWN

2264/B641
ARF6-regulated Signaling in CSF-1R-Induced Disruption of Mammary Acini.
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Expression of colony stimulating factor 1 receptor tyrosine kinase (CSF-1R) and its associated ligand, CSF, is significantly elevated in many mammary tumors. Autocrine stimulation of CSF-1R correlates with invasiveness and poor clinical prognosis. Using 3-dimensional culture models of the mammary epithelial cell line, MCF-10A, we demonstrate a role for the small GTP-binding protein, ARF6, in the regulation of chronically activated CSF-1R-induced tumorigenic phenotypes. MCF-10A cells when cultured in reconstituted basement membrane (Matrigel™), form acini composed of a single epithelial cell layer surrounding a hollow, fluid-filled lumen. In contrast, MFC-10A cells chronically stimulated with CSF form large hyperproliferative cell masses that exhibit loss of cadherin-based contacts. Furthermore, chronic stimulation with CSF leads to elevated ARF6 activation and increased CSF-1R internalization into early endosomes. The effects of internalized CSF-1R are likely mediated by ERK activation, as treatment with the MEK inhibitor, PD98059, partially rescues the wild type morphology even in the presence of chronic CSF treatment. However, when acini are subject to chronic stimulation with CSF in the presence of a dominant negative ARF6 mutant, receptor internalization is blocked, hyperproliferation and disruption of adherens junctions by CSF-1R is reversed and acini with the structural hallmarks of normal cysts are formed. These findings suggest that signaling endosomes can serve as platforms for constitutive receptor signaling during the generation of pathogenic cyst phenotypes and provide new insight into the molecular basis of epithelial glandular disruption.

2265/B642
Uptake of Cystatin by Murine Melanoma Cells.
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The cystatins belong to a family of cysteine protease inhibitors that are known to play multiple roles in cell physiology. Very little is known, however, about the interactions or uptake of cystatins by cells. Here we looked at uptake of fluorescently labeled chicken cystatin into cultured murine B16 F10 metastatic melanoma cells. We found a rapid uptake of cystatin that was apparently distinct from the uptake of Texas Red labeled dextran. Colocalization of cystatin and labeled dextran primarily occurred in the perinuclear region of the melanoma cells. We also found cystatin uptake to be independent of caveolin 1 which was detected by immunofluorescent cell staining. Several inhibitors were tested for their effects on cystatin uptake in pretreated melanoma cells. Neither blebbistatin (an inhibitor of Myosin II ATPase) nor colchicine (a microtubule inhibitor) had a dramatic effect on cystatin uptake by cells in culture. Wortmannin (a PI3-kinase inhibitor), however, decreased cystatin uptake by B16 melanoma cells. These studies show that the uptake of cystatin by melanoma cells is through a selective pathway that requires neither microtubules nor Myosin II and is also apparently independent of caveolae.

2266/B643
Relocalization of Syntaxin 1 Mediated by Endocytosis Is Important for Meiotic Cytokinesis in Fission Yeast.
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The formation of the new plasma membrane (PM) is important for cytokinesis. During fission yeast meiosis, the forespore membrane (FSM), which becomes the PM of spore, is formed within mother cell cytoplasm de novo. That is a unique type of cytokinesis and a nice model system to study de novo formation of the PM during cytokinesis because new PMs of daughter cells are separated from those of mother cells. Prior to the FSM formation, the PM t-SNARE component Psy1 relocalizes dynamically from the PM to the FSM. Here, we report that the relocalization of Psy1 is mediated by endocytosis. Psy1 was internalized after meiosis I. Photoconversion experiments using Dendra2 suggest that Psy1 is translocated from the PM to the FSM. Endocytosis during meiosis is largely dependent on F-actin, type I myosin and fimbrin. In addition, the signal induced by Mei4, a meiosis-specific transcription factor, is essential for Psy1
relocalization. The other PM proteins, Tco1 and Pmd1 were internalized during meiosis I, whereas, a P-type ATPase, Pma1, persisted on the PM, demonstrating that Psy1 is selectively internalized. Furthermore, sterol-rich membrane domain was involved in spatiotemporal regulation of the internalization of Psy1. We suggest that relocalization of exocytic t-SNARE mediated by endocytosis promotes membrane vesicle transport to form the new PM.

2267/B644

**Actin Dependent Drifts Support Infectious Endocytic Uptake of Adenoviruses.**

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Dynamic interactions of virus particles with their receptors determine lateral particle motions on the plasma membrane which is key for infectious virus entry. Here, we used human adenoviruses to explore how receptor-mediated cell surface movements are linked to endocytic uptake. Adenoviruses attach to the cell adhesion protein Coxsackie virus B Adenovirus receptor CAR, and subsequently engage with alpha v integrins, which triggers infectious dynamin-dependent endocytosis. Using live fluorescence imaging, single particle tracking and trajectory segmentation we mapped distinct movements of Adenovirus type 2 (Ad2) on the cell surface from the moment of cell binding to internalization. Three distinct motion types were identified, diffusion, drifts and confined motions on both filopodial extensions and the cell body. While CAR was necessary for Ad2 drifts, alpha v integrins reduced the drifts suggesting that switching between receptors controls cell surface movements and confinement. The drifting motions but not diffusion or confined motions required filamentous actin (F-actin) and myosin 2. Drifting motions supported infection but were not required for endosomal uptake of virus particles, as indicated by EM analysis of an Ad2 mutant (Ad2-ts1) which bound to CAR and integrins but endocytosed independently of F-actin and myosin 2. In agreement, single Ad2 or Ad2-ts1 particles drifted before dynamin-GFP recruitment and internalization. This resulted in endocytic uptake, in contrast to cases where dynamin-GFP was recruited to highly confined particles which did not lead to internalization. Unlike Ad2, Ad2-ts1 is known to fail at endosomal escape, although it has an identical surface as Ad2 capsids at 10 a resolution (Silvestry et al., JVI, May 20, 2009). Ad2-ts1 capsids are, however, more stable than Ad2 capsids as a consequence of deficient proteolytic maturation. Our data thus indicate that actin and myosin-dependent cell surface drifts trigger changes in CAR-tethered Ad2 capsids, and thereby gate dynamin-dependent endocytic uptake of virus particles, which leads to infectious viral escape from endosomes.

2268/B645

**A Quantitative, Real-time Description of the Human Immunodeficiency Virus Adhesion.**

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The fusion of human immunodeficiency virus type 1 (HIV-1) to host cells is a dynamic process governed by the interaction between glycoproteins on the viral envelope and major receptor CD4 and co-receptor on the surface of the cell. How these receptors organize at the virion-cell interface to promote a fusion competent site is not well understood. Using single-molecule force spectroscopy, we map the tensile strengths, lifetimes, and energy barriers of individual intermolecular bonds between CCR5 tropic HIV-1 gp120 and its receptors CD4 and CCR5 or CXCR4 as a function of the interaction time with the cell. at short times of contact between cell and virion, the gp120-CD4 bond is able to withstand forces up to 35 pN, has an initial lifetime of 0.27 s, an energy barrier of 6.7 kBT, and an intermolecular length of interaction of 0.75 nm. However, within 0.3 s, individual gp120-CD4 bonds undergo rapid destabilization accompanied by a shortened lifetime and a lowered tensile strength. This destabilization is significantly enhanced by the co-receptor CCR5, not CXCR4 or fusion inhibitors, which suggests that it is directly related to a conformational change in the gp120-CD4 bond. In addition using computational modeling, we simulate the interaction between a viral particle and a flexible plasma
membrane. We show that the organization and structural constraints of particle membrane associated proteins, in conjunction with the elasticity and surface tension of the cellular plasma membrane can induce an organized protein micro-domain on the plasma membrane and dictate the extent of spontaneous engulfment into the host cell.

Protein Targeting (2269 – 2287)

2269/B646
The Structural Basis of Tail-Anchored Membrane Protein Recognition by Get3.

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Targeting of newly synthesized membrane proteins to the endoplasmic reticulum is an essential cellular process. Most membrane proteins are recognized and targeted co-translationally by the signal recognition particle. However, nearly 5% of membrane proteins are 'tail-anchored' (TA) by a single C-terminal transmembrane domain that cannot access the co-translational pathway. Instead, TA proteins are targeted post-translationally by a conserved ATPase termed Get3. The mechanistic basis for TA protein recognition or targeting by Get3 is not known. Here we present crystal structures of Get3 in 'open' (nucleotide-free) and 'closed' (ADP-AlF4−-bound) dimer states. In the closed state, the dimer interface of Get3 contains an enormous hydrophobic groove implicated by mutational analyses in TA protein binding. In the open state, Get3 undergoes a dramatic rearrangement that disrupts the groove and shields its hydrophobic surfaces. These data provide a molecular mechanism for nucleotide-regulated binding and release of TA proteins during their membrane targeting by Get3.

2270/B647
Novel Regulators of ABC Transporter Biogenesis Revealed by Systematic Yeast Genetic Analysis.

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ATP-binding cassette (ABC) transporters are a superfamily of multispanning membrane proteins that utilize the energy of ATP hydrolysis to move peptides, drugs, and ions across membranes. Understanding the mechanism by which these proteins are synthesized is critical in understanding cancer drug resistance, bacterial antibiotic resistance and cystic fibrosis. We use the yeast protein required for oligomycin resistance (Yor1p) to examine the molecular mechanisms of ABC transporter biosynthesis. Yor1p functions as a pleiotropic drug pump at the plasma membrane to detoxify the cytosol of multiple toxic compounds. Deletion of a Phe residue (F670) causes Yor1p to misfold, resulting in endoplasmic reticulum (ER) retention, and degradation via the ubiquitin-proteasome pathway. Cells expressing the yor1ΔF670 allele that also contains a partial suppressing mutation, R1116T, (yor1ΔFRT) display reduced levels protein at the plasma membrane, similar to Yor1pΔF670, but show an intermediate drug resistance phenotype. Using this intermediate growth phenotype, we have developed a genetic screen to identify extragenic suppressors and enhancers with the aim of identifying novel proteins involved in trafficking, folding, and retention of ABC proteins. We used the SGA (Synthetic Genetic Array) technology to systematically disrupt all nonessential genes in cells expressing yor1ΔFRT. Next, we grew these strains in the presence of the toxin oligomycin and identified gene deletions that increased or decreased drug resistance. We found that deletion of cue1 and rpn4, components of the ER-associated degradation pathway, increased drug resistance despite the misfolding mutation. Biochemical analysis revealed that in these strain backgrounds Yor1pΔFRT is stabilized and ER-associated degradation is reduced. Conversely, the deletion of ER-vesicle
protein, Erv14p, caused inefficient ER to Golgi trafficking of Yor1p, resulting in decreased oligomycin resistance.

2271/B648
The C-Terminal Domain of the Non-Selective Cation Channels hTRPC1, hTRPC3 and hTRPC7 Determines Targeting to Intracellular Compartments.
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In keeping with their role as calcium entry channels, the TRPC (transient receptor potential canonical channel) subfamily has been proposed to reside primarily at or near the plasma membrane. However, in initial immunofluorescence studies we noted large intracellular pools for two subsets of this family, hTRPC1 and hTRPC3/7. The distribution of overexpressed hTRPC1 in COS-7 cells was restricted primarily to the endoplasmic reticulum (ER). In contrast, hTRPC3 and hTRPC7 were distributed throughout the cell, notably in the Golgi complex, plasma membrane and peripheral as well as perinuclear endosomes. Targeting to these localisations was investigated using two approaches: 1) C-terminal domain swaps and 2) generation of chimaeras of the extracellular and lumenal domain of CD8, a protein normally resident on the plasma membrane, and the C-terminal domains of hTRPC1, 3 and 7. Substitution of the C-terminal domain of TRPC3 with that of TRPC1 results in the chimaeric protein being retained in the ER. In contrast, substitution of TRPC3 C-terminal domain with that of TRPC7 does not differ from the distribution pattern of the TRPC3/7 wildtype protein and exit from the ER is not affected. In order to address the possibility that the ER distribution of the TRPC3/1 chimaera could be caused by misfolding, the CD8 reporter system was used. The distribution of both CD8-hTRPC3 and CD8-hTRPC7 resembles the endosomal pattern obtained with the full length protein including a large perinuclear pool as well as a sub-plasmalemal pool. Surprisingly, the distribution of CD8-hTRPC1 also resembles the full length hTRPC1 and is mainly retained in the ER. These observations support the hypothesis that the C-terminal domain is sufficient to target each of the TRPCs to their relative intracellular compartments.

2272/B649
Furin Is Localized in the trans-Golgi Network of Saccharomyces cerevisiae by Sorting Signals that Retain the Protein in Human Cells.
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Kex2p is a type I membrane protein whose localization to the yeast, trans-Golgi network (TGN) is required for proteolytic processing of pro-α-mating factor polypeptide into α-mating factor (αMF). Furin, a mammalian homolog, is localized to the TGN and to a lesser extent to the cell surface. Each protease is retained in high steady state in the TGN by retrieval from endosomes. Furin’s retention signals are found in its cytoplasmic tail (CT) and include the phosphofurin acidic cluster (PAC) that is bound by PACS-1 during its retrieval from the endosome. To determine if human furin localizes similarly in yeast and which CT signals may be recognized by yeast machinery, a chimeric gene (KF) was constructed to express the N-terminal, enzymatic portion of Kex2p fused to the C-terminal tail of furin. A GAL1 promoter was used to overexpress KF and KEX2 and their expression was compared in kex2Δ strains with normal and deficient vacuolar proteolysis (pep4Δ prc1Δ prb1Δ). Measures of αMF produced by KFp and Kex2p were comparable as determined by mating assay and growth inhibition but were elevated in the pep4Δ strain. Surface-specific protease activity of KFp in intact cells was 30-50% of total activity (5% for Kex2p). Immunolocalization of KFp and Kex2p showed punctate staining associated with Golgi bodies, but KFp also presented halos typical of cell surface localization. Interestingly, when the KFp PAC phosphorylation sites (S773DS775) or the main acidic motif (E776ED) were individually mutated,
nearly all mating was eliminated. Mating differences between KFp and its PAC site-specific mutants appear to be due to post-translational differences as RT-PCR analysis of mRNAs show the same degree of transcription. Additionally, mutation of a second signal in the furin tail Y759→A, possibly involved with AP-1 mediated exit from the TGN in human cells, appears to increase mating activity slightly. These findings support the hypothesis that trafficking of furin in yeast is similar to that in human cells. Surprisingly, as a PACS-1 homolog is not known to exist in yeast, it appears that phosphorylation of the PAC and the presence of the acidic cluster motif are required for TGN maintenance of KFp for αMF production and mating.

2273/B650
**Palmitoylation Regulates the Membrane Localization and Activity of Rab1a.**
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Rabs are a family of small GTPases which contribute to the regulation of intracellular trafficking by modulating vesicle budding, uncoating, motility and membrane fusion. Rabs are able to recruit specific effectors such as coat proteins, sorting adaptors and kinases to various membrane locations. Deregulation of Rab GTPases has been implicated in various diseases such as choroideremia, a progressive loss of vision caused by the degeneration of the choriocapillaris and other neurological disorders. In this study we demonstrate using the acyl-biotin exchange palmitoylation detection assay that, similar to their yeast homologue YPT1, Rab1a and Rab1b are both palmitoylated on their C-terminal extremities. Furthermore we determine using three Rab1a mutants that both of the C-terminal cysteines (C204 and C205) can be palmitoylated. Furthermore, we demonstrate through co-immunoprecipitation that DHHC-3, -5, -7, -17 and -22 are potential palmitoyltransferases responsible for Rab1a palmitoylation. Functionally, we show that palmitoylation is required to correctly localize Rab1a and Rab1b to the Golgi apparatus. Rab1b has previously been shown to recruit the Golgi-specific brefeldin a resistance factor 1 (GBF1) to Golgi membranes. We demonstrate that palmitoylation deficient Rab1 prevents the recruitment of the GBF1, a Guanine Nucleotide Exchange Factor (GEF) which plays a major role in vesicular trafficking. Palmitoylation deficient Rab1 also affected the localization of coat protein-I (COPI), a protein complex that plays an important role in ER to Golgi trafficking. Taken together, our studies suggest a major role of palmitoylation on both C-terminal cysteines for the function and localization of the small GTPase Rab1a and the correct control of vesicular trafficking from the Golgi apparatus.

2274/B651
**Calnuc Binds to LRP9 and Affects Its Endosomal Sorting.**
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Calnuc is an ubiquitous calcium-binding protein found in the cytoplasm where it binds different Gα subunits, in the Golgi lumen where it constitutes a major calcium storage pool, and outside the cell. We identified LDLR-related protein 9 (LRP9) as the first transmembrane protein shown to interact directly with Calnuc. LRP9 is a member of a new subfamily of the LDLR superfamily that cycles between the trans-Golgi network (TGN) and endosomes through a mechanism dependent on clathrin adaptor GGA proteins. The aim of the present study was to characterize the interaction between Calnuc and LRP9. Various biochemical assays showed that the N-terminus of Calnuc interacts with an arginine-rich region in the cytosolic tail of LRP9. Confocal microscopy showed that Calnuc colocalizes with LRP9 at the surface of the TGN and early endosomes. Depletion of Calnuc by small interfering RNA (siRNA) missorted LRP9 in the late endosome/lysosome compartments and enhanced its lysosomal degradation. This phenotype was rescued by the expression of siRNA-resistant wild-type Calnuc as well as cytoplasmic Calnuc, indicating that the cytoplasmic pool of Calnuc is involved in LRP9 endosomal sorting to prevent the delivery of LRP9 to lysosomes. This is the first report showing that Calnuc plays a role in receptor trafficking.
Clathrin Adaptor Complex AP-3 Regulates Early Endosomal Sorting of GPCRs.
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Sorting of G-protein coupled receptors (GPCRs) at the early endosome is required for receptor resensitization and signal attenuation. The factors that determine whether receptors enter the recycling or degradation pathways remain poorly understood. Protease-activated receptor-1 (PAR1) is a GPCR irreversibly activated by proteolysis. Hence, the termination of PAR1 signaling is mediated by direct, rapid trafficking to the lysosome and degradation. Thus, PAR1 is ideal for delineating the mechanisms required for receptor sorting at the early endosome. Unlike many GPCRs, lysosomal sorting of PAR1 occurs independent of ubiquitination and the ubiquitin-binding ESCRT components HRS and Tsg101. Instead, we have discovered that PAR1 lysosomal sorting is mediated by the clathrin adaptor complex AP-3, a heterotetrameric complex that binds to tyrosine-based sorting motifs. The AP-3 complex is known to mediate the trafficking of lysosomal membrane proteins. Activated PAR1 associates with AP-3 and co-localizes with AP-3 at the early endosome. Moreover, PAR1 contains a cytoplasmic tail tyrosine-based sorting signal YSIL, which is required for binding to the AP-3 complex. Moreover, mutation of this sorting sequence impairs PAR1 degradation by delaying receptor entry into the intraluminal vesicles of the multi-vesicular endosome, but has no effect on receptor internalization. The location of this tyrosine-based sorting sequence in the PAR1 cytoplasmic tail is conserved in many other GPCRs, indicating that this could be an alternative mechanism for sorting of GPCRs into the degradation pathway. These results demonstrate for new a role for the AP-3 complex and a tyrosine-based lysosomal targeting sequence in GPCR degradation.

Arrestin-2 Interacts with the HRS/STAM-1 Complex to Regulate CXCR4 Sorting into the Degradative Pathway.
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The chemokine receptor CXCR4, a G protein-coupled receptor (GPCR), and its cognate ligand stromal cell-derived factor 1a (SDF-1α; a.k.a. CXCL12) form a major receptor/chemokine signaling axis in normal mammalian physiology and disease. Dysregulation of CXCR4 signaling and trafficking has been implicated in several human pathological conditions including cancer, cardiovascular disease and WHIM syndrome. However, the molecular mechanisms that control CXCR4 signaling and trafficking remain poorly understood. In these studies, we focused on understanding how CXCR4 trafficking is regulated in the endocytic pathway. We previously showed that non-visual arrestin-2 localizes to endosomes and plays an important role in sorting activated CXCR4 from endosomes into the lysosomal degradative pathway. Arrestins are endocytic adaptor molecules that facilitate clathrin-mediated endocytosis of GPCRs however, their function in endocytic sorting of GPCRs into the degradative pathway remains poorly understood. Here, we show that arrestin-2 interacts directly with STAM1, but not to the closely related protein STAM2, using a combination of co-immunoprecipitation and GST fusion-protein interaction approaches. Both STAM1 and 2 are components of ESCRT-0, a multi-protein complex containing HRS that recognizes and recruits ubiquitinated cargo to multivesicular bodies and the lysosomal degradative pathway. Moreover, arrestin-2 co-localizes with STAM-1 on EEA1 positive endosomes together with internalized CXCR4, as assessed by confocal immunofluorescence microscopy studies. We mapped the arrestin-2 binding site on STAM1 to the GAT (GGA and TOM1 homologous) domain via truncation mutagenesis. Interestingly, expression of a GAT domain minigene blocks the interaction between STAM1 and arrestin-2 and enhances agonist-promoted degradation of CXCR4. Thus, we have identified a novel interaction between arrestin-2 and STAM-1 and our data suggest that this interaction is important for proper regulation of activated CXCR4 sorting into the lysosomal degradative pathway.
2277/B654
*TBRIII Directs Clathrin-Mediated Endocytosis of TGFβ Receptors.*

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Transforming Growth Factor Beta (TGFβ) signalling is involved in both normal developmental processes, such as organogenesis, and pathological disorders, such as cancer and fibrosis. There are three prototypical TGFβ receptors: TβRI, TβRII, and TβRIII. While the role of TβRII and TβRI in TGFβ signal propagation has been heavily investigated, the contribution of TβRIII to TGFβ signalling is not well understood. At the cell surface, all three receptors can be internalized by both clathrin-mediated or clathrin-independent, membrane-raft dependent endocytosis. Interestingly, the endocytic route of the receptors plays a direct role in TGFβ-dependent Smad signal transduction; receptors internalized via clathrin-coated pits activate Smad signalling, whereas receptors directed into membrane rafts are targeted for degradation. The objective of the present study is to evaluate the contribution of TβRIII to TβRII membrane partitioning, endocytosis, and Smad signal transduction. Using subcellular fractionation, we show that TβRIII alters the endocytosis of TβRII by recruiting TβRII to non-membrane raft fractions. Similarly, an antibody-chase immunofluorescence approach indicates that the over-expression of TβRIII affects intracellular trafficking of TβRII by recruiting TRβII to the early endosome. Using [125I]-labelled TGFβ to follow cell-surface receptors, we show that over-expression of TβRIII also extends the receptor half-life of TβRII. Finally, siRNA to TβRIII showed that loss of TβRIII has direct consequences on TGFβ-dependent Smad signalling. In this study we show that TβRIII increases clathrin-mediated endocytosis of TβRII, and hence TGFβ signalling. As numerous pathologies show aberrant activation of TGFβ signalling, this study illustrates that TβRIII may represent a novel therapeutic target for decreasing TGFβ signalling.

2278/B655
*Differential Sub-cellular Localization, Internalization, and Recycling of RET Receptor Tyrosine Kinase Isoforms.*

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The RET receptor tyrosine kinase is essential for development of the kidneys and enteric nervous system. RET is alternatively spliced at the 3’ end resulting in the co-expression of three isoforms that differ in 9, 43, or 51 C-terminal amino acids, respectively. The two most highly expressed isoforms, RET9 and RET 51, induce unique autophosphorylation patterns on intracellular tyrosine residues, differentially bind downstream signalling proteins, regulate distinct patterns of gene expression, and possess different intrinsic abilities to cause cellular transformation and differentiation. We hypothesize that differential sub-cellular localization and trafficking of RET isoforms plays a role in mediating their differences in vivo. Here, we present a comprehensive study of the differential sub-cellular localization and trafficking of RET isoforms in primary rat enteric neurons and model human cell lines. We have observed that RET9 maturation is less efficient relative to RET51 resulting in an accumulation of RET9 in the Golgi and a higher relative expression of RET51 on the plasma membrane. Post-activation, RET9 present at the cell surface is targeted through endosomes directly to the lysosome. Although RET51 is internalized more rapidly than RET9, a portion of it was found to recycle back to the plasma membrane. Recycling prevents efficient degradation of RET51, allowing for prolonged activation of downstream signalling cascades by this molecule, relative to RET9. Together, these results suggest that differential sub-cellular localization and trafficking influence the functional differences observed between RET9 and 51 in vivo.

2279/B656
*Yeast α-Arrestins Regulate Intracellular Trafficking of Nutrient Permeases.*
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β-arrestins regulate the desensitization and endocytosis of G-protein coupled receptors. Little is known about the structurally related α-arrestins found in many eukaryotes, including mammals. Yeast possess nine α-arrestins, three of which mediate protein endocytosis. We show that the closely related yeast arrestins Aly1 and Aly2 regulate intracellular trafficking and exocytosis of membrane proteins. This novel function suggests that arrestin family members act as global regulators of protein trafficking. Aly1 and Aly2 modulate yeast resistance to rapamycin, indicating that they function in nutrient signaling. These α-arrestins interact with the nutrient permease Gap1 and increase Gap1 levels in intracellular compartments and at the PM. Aly1 and Aly2 localize to endocytic compartments, co-localize with clathrin, and co-purify with clathrin and adaptor complex proteins. This is the first evidence that α-arrestins, like β-arrestins, interact with the membrane trafficking machinery. Aly1 and Aly2 act in distinct Gap1 trafficking pathways and are differentially regulated. Aly2 interacts with and requires the nitrogen permease re-activator kinase Npr1, which promotes Gap1 PM localization via an unknown mechanism. Aly1 is positively regulated by the protein phosphatase calcineurin and must be dephosphorylated for its optimal regulation of Gap1. Aly2 acts on a pool of Gap1 that is recycled from multi-vesicular bodies back to the Golgi; Aly2 overexpression fails to promote Gap1 PM localization in lst4Δ cells where the recycling pathway is crippled. Aly1 overexpression promotes Gap1 PM localization in lst4Δ cells, indicating that Aly1 acts at an earlier stage in Gap1 trafficking. In higher eukaryotes, arrestins are critical links between extracellular events such as receptor-ligand binding and intracellular signaling. Aly1 and Aly2 similarly link extracellular nutrient cues to intracellular signaling in yeast.

2280/B657

Amino Acid Sensing by Gap1p Contributes to its Intracellular Sorting in S. Cerevisiae.

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The general amino acid permease Gap1p of Saccharomyces cerevisiae is subject to regulated trafficking in response to amino acids. Gap1p is active at the plasma membrane when amino acids are absent. An increase in amino acids leads to direct sorting of Gap1p from the trans-Golgi to the vacuole, preventing import of toxic amounts of amino acids into the cell. Under conditions of high extracellular amino acids most of the intracellular Gap1p en route to the vacuole is located in the membrane of the multivesicular endosome (MVE). If amino acid levels fall Gap1p can be mobilized from the MVE to the plasma membrane. The mechanisms by which amino acids are sensed and intracellular sorting of Gap1p is regulated are poorly understood. We have identified a substrate specificity mutation of Gap1p, A297V, which blocks transport of positively charged amino acids such as lysine and arginine, while preserving the ability to transport other amino acids, including glycine, phenylalanine, and threonine. We have designed experiments to test the sorting of Gap1p A297V and find that this mutant escapes the MVE membrane and returns to the plasma membrane in the presence of positively charged amino acids. These results indicate that Gap1p can function as its own amino acid sensor to direct sorting to the plasma membrane or retention in internal compartments.

2281/B658

The Cbl RING Finger Tail Regulates Sorting Endosome Fusion.

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The ubiquitin ligase c-Cbl controls epidermal growth factor receptor (EGF-R) signaling by enhancing receptor ubiquitination, downregulation, and degradation. Cbl residues 1-434 are the minimal evolutionarily conserved sequences sufficient to regulate EGF-R. They encompass a tyrosine kinase-binding domain, linker region, RING finger (RF), and a subset of the RF tail amino acids 420-436. Functional roles for RF tail residues I429, V430, D432, and P433 were suggested,
but not demonstrated, by a solved Cbl/UbcH7 crystal structure. Our objective was to use alanine scanning mutagenesis and structure/function studies to identify RF tail amino acids that regulate EGF-R fate, and then to determine how they do it. Constructs encoding Cbl RF tail alanine substitution mutants were generated. Wild type and mutant Cbl proteins were expressed transiently or inducibly in HEK 293 and COS-7 cells; the cells were then assayed for EGF-R trafficking and degradation. Trafficking events were followed by fluorescence localization of GFP-tagged Cbl proteins, EGF and markers of endosomal compartments in fixed and live cell studies. We determined that the identity of I429, V430, D432, and P433 is not critical for Cbl-enhanced EGF-R downregulation and degradation. However, the integrity of V431 and F434 is crucial. Protein biochemistry and fluorescence imaging studies revealed: 1) that Cbl mutant V431A fails to effect the ubiquitin-dependent degradation of hSprouty2 and compromises EGF-R internalization; and 2) that mutant F434A compromises EGF-R degradation later in the endocytic trafficking pathway, stabilizing phosphorylated Hrs and retarding sorting endosome fusion. Our identification of the post-internalization trafficking checkpoint controlled by Cbl, and our development of a testable mechanistic model for Cbl's regulation of endosome fusion at that site, provide a new focus for studies of EGF receptor regulation. Supported by American Cancer Society grant RSG-03-046-01-GMC and NIH award RO1CA109685(to N.L.L.).

2282/B659
The Cation-Dependent Mannose 6-Phosphate Receptor Is Ubiquitinated and Palmitoylated for Its Sorting In Endosomes.

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Background: The cation dependent mannose 6-phosphate (CD-MPR) constitutively cycles between the TGN and the endosomes to deliver newly synthesized mannose 6-phosphate tagged lysosomal enzymes from the TGN via endosomes to the lysosomal compartment. Sorting of the CD-MPR within the endosomal compartment has been shown to require the S-palmitoylation of a cystein residue in its cytoplasmic tail to avoid delivery of the receptor itself to the lysosomes.

Methodology/Principal Findings: In this work, we demonstrate that this reversible post-translational modification allows the transient association of the CD-MPR with detergent resistant membrane (DRM) subdomains which are not localized at the plasma membrane (PM). Additionally, using a FLAG-tagged ubiquitin approach we could show that the CD-MPR can be ubiquitinated. The ubiquitination of the CD-MPR also occurs at the PM and is not required for the internalization of the CD-MPR from the PM whereas de-ubiquitination occurs in early endosomes.

Conclusions/Significance: Altogether, these results suggest that the retrieval of the CD-MPR from the endosomal compartment to the TGN requires the association of the receptor with DRM subdomains located within the early endosomes via S-palmitoylation. Additionally, an ubiquitin ligase activity for the CD-MPR exists at the PM, suggesting that ubiquitination might be involved quality control mechanisms of non functional receptors leading to lysosomal degradation.

2283/B660
Purification and Focused Proteomic Analysis of Lipid Rafts Which Mediate Human Vascular Smooth Muscle (VSM) Ca$^{2+}$-sensitization.

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Hypercholesterolemia is a major risk factor for cardiovascular events. Although Ca$^{2+}$-sensitization of VSM contraction contributes to abnormal VSM contractions such as vasospasm, its relation with cholesterol has yet to be clarified. We found that sphingosylphosphorylcholine (SPC) / Src...
Family Tyrosine Kinase (Src-TK)/Rho-kinase (ROK) pathway mediates the Ca\(^{2+}\)-sensitization, and SPC indeed induces severe vasospasm in vivo. Interestingly, the SPC-induced Ca\(^{2+}\)-sensitization of human VSM showed positive and inverse correlations with the serum total cholesterol (CHOL) and LDL-CHOL, respectively. CHOL depletion not only destroyed lipid rafts but also inhibited the SPC-induced Ca\(^{2+}\)-sensitization, without affecting Ca\(^{2+}\)-dependent contraction, suggesting an important role of lipid rafts in abnormal VSM contraction. Understanding the mechanism(s) by which lipid rafts promote Ca\(^{2+}\)-sensitization of human VSM requires the elucidation of lipid raft protein composition. As a first step to this approach, we succeeded for the first time in purifying lipid rafts from human VSM by sucrose density gradient ultracentrifugation, which were confirmed by the presence of raft marker proteins such as caveolin-1 and flotillin-1. Meanwhile, focused proteomics has been considered as a strong tool to elucidate molecular and cellular pathogenesis, and to screen for possible molecules which are responsible for dysfunctions and abnormal intracellular signaling in the diseased states. Using mass spectrometry (MALDI TOF-MS), proteomic analysis was performed to compare the protein compositions between lipid raft and non-lipid raft membrane fractions. The ongoing studies have identified so far previously unreported novel raft-localized proteins, in addition to the known proteins, including lipid- or GPI-anchored proteins and membrane proteins. We are attempting to accumulate functional data to suggest that some novel signaling molecules contribute to an SPC/Src-TK/ROK pathway leading to the VSM Ca\(^{2+}\)-sensitization and vasospasm.

2284/B661
Effects of the Novel Compound Sortin2 on the Endomembrane System of Arabidopsis thaliana.
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Sortin2 is a synthetic low mass compound that was identified by a chemical genomics approach. This compound alters targeting to vacuole of a soluble vacuolar protein marker in yeast and plants. Sortin2 also alters vacuole morphology in plants. Considering the complexity of the endomembrane system the use of chemical compounds such as Sortin2 as a valuable probe would be a powerful tool. Therefore we are now in the process of identifying the cellular pathways targets of Sortin2. The objective of this study is to analyze the impact of Sortin2 on trafficking to different compartments within the endomembrane system of A. thaliana in order to understand its mode of action. The targeting and localization of different GFP-proteins markers were analyzed by confocal microscopy on Sortin2-treated transgenic Arabidopsis seedlings. The effect of Sortin2 was also compared to other known drugs that alter the endomembrane system. Our results show that Sortin2 alters the localization of plasma membrane proteins that traffic to endosomes causing that they localize in intracellular compartments of bigger size than endosomes that are similar to vacuole. These compartments are morphologically different than Brefeldin a (BFA) bodies. Furthermore Sortin2 treatment inhibits the accumulation of plasma membrane proteins into BFA bodies suggesting that Sortin2 has an effect on the endocytic trafficking. Sortin2 does not affect targeting of protein markers of Golgi apparatus and endoplasmic reticulum protein markers. The effects of Sortin2 are different to others drugs that alters the endomembrane system such as wortmanin, endosidin1 and Exo-1. Sortin2 impacts the endomembrane system of Arabidopsis thaliana in a very specific manner therefore we believe that it would be a useful tool to dissect trafficking mechanisms that would be difficult to study due to the complexity and redundancy of the endomembrane system in plants. Funding Fondecyt 11080240, Chile.

2285/B662
Probing Tonoplast Protein Trafficking with Chemical Genomics.
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The plant tonoplast is essential for the storage of metabolites and hormones, the sequestration of ions and the maintenance of plant cellular turgor. All of these tonoplast functions are regulated by
integral membrane proteins, and yet very little is known about the mechanisms for regulating the trafficking of membrane proteins to the tonoplast. Our goal is to identify the trafficking machinery for tonoplast proteins in *Arabidopsis thaliana* using a Chemical Genomic approach. Chemical Genomics is the use of small molecules to modify the activity of certain proteins or pathways. Gravacin is a small molecule that was identified in a Chemical Genomics screen for compounds that affected gravitropism and vacuole biogenesis in Arabidopsis (Surpin et al. 2005 P.N.A.S. 102:4902). This compound inhibits the delivery of a tonoplast marker, a GFP fused to the delta-Tonoplast Intrinsic Protein (GFP-δ-TIP), to the tonoplast. We are currently characterizing mutants with altered sensitivity to this inhibitor in order to understand its site of action in trafficking pathways. We have used map-based cloning and next generation sequencing to identify genetic lesions in one of the Gravacin-hypersensitive mutants. Candidate genes, including a gene encoding a pleiotropic drug resistant protein, are being analyzed for potential roles in protein trafficking. In addition, a new screen was initiated and has yielded novel inhibitors of the pathway. The specificity of these inhibitors for integral membrane proteins underscores a multiplicity of pathways and the specificity of the machinery that targets membrane proteins to the vacuole.

2286/B663
Identification of a YxxL Motif-Containing Transmembrane Protein as a Putative Receptor of the Major Virulence Factor Cysteine Protease in the Enteric Protozoan *Entamoeba histolytica*.

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The enteric protozoan parasite *Entamoeba histolytica* is one of the most widespread and clinically important parasites, causing both serious intestinal (amoebic colitis) and extraintestinal (amoebic liver abscess) diseases. *E. histolytica* is characterized by its extraordinary capacity to invade and destroy human tissues. Secreted cysteine proteases (CP) are the main lytic molecules and 98% of CP activity is attributable to three CPs, CP1, CP2, and CP5. Among them, CP5 is not expressed in a non-pathogenic *Entamoeba* species, *E. dispar*, thus considered to be one of the key determinants of the virulence. Similar to CPs in other organisms, e.g., cathepsins, amoebic CPs also have a role as digestive enzymes in lysosomes. However mechanisms of CP targeting and secretion remains poorly understood. We previously demonstrated that the amoebic retromer complex is involved in CP trafficking and the putative receptor-binding component, Vps35, was conserved in the amoebic retromer complex. This observation led us to assume the presence of a receptor-mediated CP transport system in *E. histolytica*. To get more insights into molecules involved in CP trafficking, we attempted to biochemically hunt for CP-interacting molecules. An *E. histolytica* transformant that expressed HA-tagged CP5 was established and CP5-HA-associated proteins were immunoprecipitated. A 100-kDa protein was specifically precipitated from the CP5-HA overexpressor. As a result of mass spectrometric analysis, the protein was identified as a protein with the signal sequence and a single carboxy-terminal transmembrane region, followed by a 19-amino acid cytoplasmic tail containing adaptor protein-binding YxxL motif at the very C-terminal end of the protein. In *E. histolytica* genome database, there are other 10 genes with similar size and structure to this molecule. We named these proteins as cysteine protease binding protein family; CPBF and CP5-HA-binding protein as CPBF1. In our previous phagosome proteome analysis, three CPBFs including CPBF1 were detected. These data suggest that CPBFs are involved in the sorting of hydrolases by AP complex-mediated transport, possibly TGN-to-endosome trafficking.

2287/B664
Xenocin Export in *Xenorhabdus nematophila*.

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*Xenorhabdus nematophila*, an insect pathogenic bacterium resides as a symbiont in the gut of a soil nematode *Steinernema carpocapsiae*. The bacterium produces potent toxins to kill its insect
prey. Both the bacterium and the nematode feed upon the nutrient rich larval carcass and undergo several cycles of growth and development. In order to keep the dead larva free from competing and putrefying bacterial species, X. nematophila produces a variety of microbicidal peptides and protein. The focus of this study is a bacteriocin xenocin, produced by X. nematophila in response to SOS signals. Xenocin has endoribonuclease activity and was found to be active against both Gram positive and negative bacteria. Bacteriocins are generally released in the surrounding media by lysis of the producer cells. The lysis occurs with the help of a lysis inducing protein, encoded in the same operon or near the bacteriocin encoding genes. Screening of X. nematophila genome revealed no homologous sequence near the xenocin operon. Preliminary investigations show an atypical pathway of xenocin export in X. nematophila. Inability of a flagellar secretary (flhA) mutant to secrete xenocin demonstrated that the latter is exported through flagellar secretory pathway.

**Imaging Technology (2288 – 2307)**

2288/B665

**Monomeric Photoconvertable Fluorescent Protein Variants Produced by Directed Evolution for Brightness and Efficient Photoconversion.**

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The use of photoconvertible (PC) fluorescent proteins (FPs) enables researchers to selectively highlight a subcellular population of a fusion protein of interest and track its dynamics in vivo. Another important application of PCFPs is subdiffraction-limit imaging, which relies on the high resolution localization of individual photoconvertible probes (Bates 2008). In an effort to enrich the arsenal of PCFPs and overcome the limitations imposed by the oligomeric structure of the natural PCFPs, we set out to design and subsequently evolve a new monomeric PCFP. First, all of the known PCFPs (i.e., EosFP, Dendra2, Kaede and KikGR (Ando 2002, Wiedenmann 2004, Gurskaya 2006, Tsutsui 2005)) were aligned to find the consensus for each amino acid (Dai 2007). Amino acids of >50% consensus were chosen for the new protein; for non-consensus and poorly conserved consensus residues, mTFP1 (Ai 2006) and mWasabi (Ai 2008) sequences were used as a “guide”, while making sure that monomerizing mutations are not removed in the consensus design. The designed gene was synthesized (Bang 2008) and, when expressed in E.coli, was found to exhibit green fluorescence that gradually switched to red after exposure to sunlight for 1-2 hours. We subjected this first-generation FP (named GR1) to a combination of random and targeted mutagenesis and screened libraries for efficient PC using a custom built ‘PC chamber’ in which a 10 cm Petri dish is evenly illuminated with light from 594 individual 405 nm LEDs. Following more than 15 rounds of library creation and screening, we settled on an optimized version, known as GR2, that has 8 mutations relative to GR1. Key features of GR2 include a 1.2-fold brighter red species, 1.5-fold higher PC efficiency, and dramatically improved chromophore maturation in E. Coli. relative to GR1. The monomeric status of GR2 has been demonstrated by gel-filtration chromatography, as well as the functional expression of GR2-β-actin fusion in Hela cell. Live cell characterization of GR2 is ongoing, but preliminary experiments indicated that GR2 is well suited for rapid PC (~1 s) of protein sub-populations and subsequent tracking of their diffusion dynamics in living cells. HH and NS contribute equally to this work.

2289/B666

**Choosing the Right Photoactivatable Fluorescent Protein for the Job.**

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Optical highlighters have changed the way we use fluorescence to visualize cellular dynamic movement. However, the highlighters have their own dynamic properties that can influence our biological measurements. Here we show that the power required to photoactivate ensembles of
commonly used highlighter molecules varies over several orders of magnitude. The level of power depends upon the chemical mechanism of activation, and higher levels of power are needed to activate photoactivatable fluorescent proteins (PAFPs) that release photons slowly. PAFPs with fast photon outputs are better able to capture rapid transients, and yield higher molecular localization precision in superresolution imaging such as photoactivation localization microscopy (PALM). In contrast, PAFPs with slower photon outputs are better suited for producing stable variations in contrast and longer time scale tracking. Surprisingly, increasing the activation power cannot significantly increase the activation speed of slower emitting PAFPs. These data indicate accurately capturing dynamic events with multiple labels in living cells is not as simple as choosing spectrally distinct PAFPs.

2290/B667
A Simple Method to Photoconvert and Track Discreet Protein Pools on Standard Wide-Field Mercury Arc-Based Microscopes.
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Green fluorescent protein (GFP) and other FP fusions have been extensively utilized to track protein dynamics in living cells. Recently, development of photoactivatable, photoswitchable and photoconvertible fluorescent proteins (PAFPs) has made it possible to investigate the fate of discreet subpopulations of tagged proteins. Initial limitations to their use (due to their tetrameric nature) were overcome when monomeric variants, such as Dendra, mEOS, and mKikGR were cloned/engineered. Here, we report that by closing the field diaphragm, selective, precise and irreversible green-to-red photoconversion (330-380 nm illumination) of discreet subcellular protein pools was achieved on a wide-field fluorescence microscope equipped with standard DAPI, fluorescein, and rhodamine filter sets and mercury arc illumination within 5-10 seconds. Use of a DAPI-filter cube with long-pass emission filter (LP420) allowed to observe and control the photoconversion process in real time. Following photoconversion, living cells were imaged for up to 5 hours often without detectable phototoxicity. We demonstrate the feasibility of this technique using Dendra2 and mEOS-tagged proteins with low (histone H2B), medium (gap junction channel protein connexin 43), and high dynamic cellular mobility (α-tubulin; clathrin light chain) as examples. Efficient, irreversible green-to-red photoconversion of portions of cell nuclei, gap junctions, microtubules and clathrin-coated vesicles was achieved. Tracking over time allowed elucidation of the dynamic live-cycle of these subcellular structures. We demonstrate the practicability of an efficient and relatively inexpensive method to photoconvert and track discreet subpopulations of protein pools in living cells using Dendra2 and mEOS as monomeric, photoconvertible PAFP representatives. The advantage of this technique is that it can be performed on a standard relatively inexpensive wide-field fluorescence microscope with mercury arc illumination. Together with previously described laser scanning confocal microscope-based photoconversion methods, this technique promises to further increase the general usability of photoconvertible PAFPs to track the dynamic movement of cells and proteins.

2291/B668
Multicolor Single Particle Tracking of Lipids and Membrane Proteins with Quantum Dots.
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Quantum dots (Qdots) are fluorescent nanoparticles that have far superior signal intensity and signal stability compared to more conventional fluorescent molecules. We find that imaging with Qdots can easily be extended to the simultaneous visualization of up to four different molecular species at single molecule sensitivity and millisecond time integration. We are applying this technique for studying the spatial and temporal nano-organization of various combinations of lipids and proteins in the cellular plasma membrane. We are in particular interested in applying multicolor single particle tracking for a comparative analysis of the diffusion characteristics of lipid raft markers in the plasma membrane of mouse fibroblasts.
Combination Labeling and Spectral Imaging,(CLASI): A Method to Greatly Expand the Number of Distinguishable Fluorescent Labels in a Single Image.
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The number of fluorescent proteins, organic fluorophores, and inorganic fluorescent biomarkers is ever increasing. In addition, modern methods of ultra high-resolution fluorescence microscopy allow characterization of the precise spatial arrangements of complex macromolecular structures within cells. However, the ability to unambiguously distinguish more than a few different labels in a single fluorescence image is severely hampered by the excitation cross-talk and signal bleed-through of fluorophores with highly overlapping excitation and emission spectra. Here, we report the development of a fluorescence labeling, imaging, and analysis method to greatly expand the number of identifiable labels in a single image. The CLASI method involves labeling targets with specific combinations of fluorophore reporters. Commercially available microscopes with spectral detection capabilities are used to image the combinatorially-labeled specimens. Novel computational algorithms are used to analyze spectrally-recorded image data. We have developed a linear unmixing algorithm constrained to identify specific combinations of fluorophores. Our novel algorithm allows the concatenation of spectral data acquired with several different excitation wavelengths, and a goodness-of-fit is reported for each spectral combination, either in every pixel or for every object identified in the image. As a proof-of-principle experiment, we have imaged mixtures of E. coli labeled with combinations of organic fluorophores. Using fluorescence in situ hybridization with oligonucleotide probes specific for 16S rRNA sequences, we demonstrate that we can distinguish 120 differently labeled microbes in a mixture using binary combinations of 16 fluorophores. Supported by a grant from the Sloan Foundation. AMV acknowledges support from an NRSA (NIH F-31) pre-doctoral fellowship from the National Institute of Dental and Craniofacial Research.

Modulation Tracking of Cellular Adhesions in 3D Collagen Matrices.
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Cell mobility, proliferation, and cell adhesions are fundamental for cancer metastasis, wound healing, stem cell differentiation, and development. The dynamics of protein interactions and cell migrations have been extensively studied in the 2D environment. We have developed methods applicable to 2D raster scan images (RICS and scanning FCS) that have given information about the formation of complexes responsible for cell adhesion and migration. It is now established that cell movement in 3D matrices and in tissues have different dynamics. In 3D the search of the adhesions site with the extracellular matrix is a daunting problem. Cells make only few transient contacts and the entire cell moves in 3D going out of focus in conventional confocal or 2-photon excitation microscopy. There is no simple way to use the raster scan fluctuations techniques in the 3D environment that were successful in determining protein complexes and interaction in 2D. We have developed an imaging method that produces high resolution 3D images with time resolution comparable to the raster scan methods used in 2D. The method is based on a tracking-imaging principle. Using this approach we are able to follow the aggregation of proteins in the point of contacts between cell filopodia and the extracellular matrix.

Automated Kinetic Characterization of Intracellular Single-molecule Trafficking.
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BACKGROUND Single molecule imaging using GFP-based bright fluorescent markers allows
direct visualization of the structural dynamics of proteins of interest while they perform their
functions. This enables new tools to investigate the sub-molecular mechanism of intracellular
trafficking. CHALLENGES Accurate tracking of multiple objects is required to quantitatively
characterize the kinetic behavior and interaction of single-molecular “objects” in a movie. This is a
challenging task due to complex object dynamics (varying object speeds, directions, contrasts
and morphologies) imaging noise and sample obscurations. APPROACHES We have developed
a fully automated, highly robust and flexible tracking method called “soft tracking” generating
tracks for our kinetic characterization tool. We separated object movements into multiple motion
energy channels. Soft tracking performs motion guided self-checking where relative motions
derived from inter-frame tracking match pairs are checked with the independently generated
motion energy channels to resolve any ambiguous tracks. STUDIES The Objective of this study is
to validate the performance of the motion guided soft tracking. Our Hypothesis is that our
automated method achieves similar performance to the best manual method. The test movie
Data sets include (1) single molecules trafficking study of a ligand-receptor complex in growth
cone of the chick dorsal root ganglion cell where objects move in different (often oppose)
directions; (2) mseGFP-labeled TrkA receptor for a translocation study where objects have
varying speed and contrast. The manual track Truth is created independently by two analysts and
discrepancies are resolved through review with the group. We Test the hypothesis using
detection and tracking accuracy metrics. RESULTS The results show high tracking sensitivity (>90%)
and low object tracking error (<10%). CONCLUSIONS We conclude that the hypothesis is
supported with statistical significance. We believe the technologies could standardize quantitative
single-molecule trafficking characterization.

2295/B672
Quantitative Analysis of Protein Proximity by Fluorescence Microscopy.
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Fluorescence resonance energy transfer (FRET) between two fluorophores is a function of their
degree of separation. This property is widely exploited experimentally to create sensors of
biological activity and monitor changes in protein structure. FRET is also applied in living cells to
measure the proximity of proteins within a complex that is tagged with a pair of fluorescent
proteins. However this last application is hampered by the lack of a statistically robust
methodology, unresolved conflicts among different approaches and a dearth of empirical data
that correlates a FRET value with distance in vivo. In our previous work we developed an intuitive
FRET metric, FretR. Here, we compare FretR with four other prevalent FRET indices. We
measured FRET in a benchmark set of proteins where the distance between YFP and CFP is
known. The benchmark set included: YFP-(Proline)n-CFP, five soluble proteins of known
structure tagged with YFP and CFP and three variants of Spc110 tagged with YFP-(Proline)n-
CFP. FretR values decreased with increasing distance and were independent of both the
abundance of YFP in the cell and the extent of YFP and CFP fluorescence spilling over into the
FRET channel. FretR values above 2.0 corresponded to points in a protein complex that were
within 30 Å. Values from 1.5 -2.0 corresponded to distances from 30 - 55 Å. This study used the
program FRETSCAL to automate all stages of image analysis. FRETSCAL is an integrated
collection of Matlab scripts that reproducibly screened, selected and analyzed hundreds of areas
of interest from images of thousands of cells. FRETSCAL is available from the Yeast Resource
Center (yeastrc.org) for non-commercial use. We conclude that FretR in combination with
FRETSCAL can be used to probe the organization of protein complexes and provide information
about the distances between proteins in a complex.

2296/B673
STED Nanoscopy in the Living Cell.
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By using Stimulated Emission Depletion (STED), far-field fluorescence microscopic images with a resolution far beyond the diffraction limit can be obtained. To this end, the excitation beam is overlaid with a doughnut-shaped, redshifted STED beam, which switches off the fluorophore's ability to fluoresce. Since the off-switching is only taking place in the outer region and not in the very center of the excitation spot, this gives rise to a diffraction-unlimited effective excitation. By scanning the nanosized effective excitation spot through the sample, images can be acquired with a sub-second framerate, which is especially beneficial for live-cell imaging. Here we demonstrate the application of STED microscopy for imaging the interior of living mammalian cells and tissue. Utilizing fluorescent proteins, we imaged different cellular organelles and elements of the cytoskeleton with a lateral (x,y) resolution of below 50 nm, corresponding to a 4-fold improvement over that of a confocal microscope, meaning a 16-fold reduction in the focal spot cross-sectional area. Likewise, an axial resolution of about 150 nm was achieved, which corresponds to an improvement of a factor of 3 compared to confocal imaging. By time lapse STED imaging of YFP-positive hippocampal neurons in organotypic slices nanoscopic videos of structural changes in living tissue were also obtained. As an alternative to autofluorescent proteins we employed genetically encoded protein tags which can selectively bind modified organic dyes. The commercially available Snap-Tag (NEB) was used as a fusion protein, which specifically forms a covalent bond with benzylguanine-substituted dyes. By using tetramethylrhodamine or Oregon Green to label fusionproteins with connexin-43, a resolution of down to 40 nm in the focal plane was observed in living mammalian cells. Also, movies were obtained, showing the movement of connexin-43 clusters across the cell membrane with nanoscale resolution. We show that by employing continuous wave lasers an economic and easy-to-handle STED microscope capable of live cell imaging can be built. This greatly expands the scope of light microscopy in cell biology.

2297/B674
DIC Microscopy with High Speed Switching Shear Directions and Its Applications.
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A standard DIC (differential interference contrast) microscope shows the two-dimensional distribution of optical path gradients encountered along the shear direction. Thus contrast of DIC images is not symmetrical and varies proportionally with the cosine of the angle made by the azimuth of the object and the direction of wavefront shear. It is therefore necessary to examine unknown objects at several azimuth orientations. To overcome the limitations of available systems, we have built an assembly, which allows the bias and shear directions to be switched rapidly without mechanically rotating the specimen or the prisms. The assembly consists of two standard Nomarski or Wollaston prisms with liquid crystal 90-degree polarization rotator in between. When the polarization rotator is in OFF state, the total shear direction of the assembly is at +45-deg to the shear direction of the first prism. If the polarization rotator is in on state, the total shear direction is at -45-deg to the shear direction of the first prism. Thus, when one would switch the polarization, the shear direction would be rotated by 90-deg. We added one assembly to the illumination path and another one to the imaging path of the standard Olympus BX-61 microscope. Also one variable liquid crystal retarder was installed into the microscope in order to change a bias. Using MATLAB we developed software for setup control and image processing. Four raw DIC images at two orthogonal shear directions and two inverse biases are captured and processed within a second. Then the refractive index gradient image is displayed on a computer screen. The orientation-independent DIC data obtained can also be used to compute the quantitative distribution of specimen phase or to generate enhanced, regular DIC images with any desired shear direction. The OI-DIC system can be combined with other techniques, such as fluorescence and polarization microscopy. The combined DIC-Pol system will yield two complementary phase images of thin optical sections of the specimen: distribution of refractive index gradient and distribution of birefringence due to structural or internal anisotropy of the cell structure.
2298/B675

A Closer Look at the Dichroic Beamsplitters - Their Effect on Resolution and Image Quality in Fluorescence Microscopy.

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Fluorescence microscopy is routinely used for the study of biological samples. One of the key enablers of a modern fluorescence microscopy instrument is the dichroic (or dichromatic) beamsplitter, also called a mirror, that can selectively transmit as well as reflect desired wavelengths of light. However, if a dichroic mirror is not suitably designed and manufactured then significant optical aberrations may be introduced, specifically in a beam that is reflected off such a dichroic. For example, when reflecting the emission channel in an imaging setup, a non-flat dichroic can appreciably change the distribution of the signal in and near the detector plane thereby changing the point spread function (PSF) of the imaging setup. Therefore resolution of such a microscope is compromised affecting the overall image quality. In the excitation light path, a non-flat dichroic can make it impossible to achieve desired illumination of the sample in demanding techniques such as Total Internal Reflection Fluorescence (TIRF) microscopy or structured illumination instruments. Despite the need for appropriate dichroics, their flatness requirements remain poorly understood and vaguely specified in fluorescence microscopy. Therefore, a design measure is needed to ensure that dichroics of sufficient flatness are used in different fluorescence microscopy applications. We investigate this issue both theoretically and experimentally, using a custom designed experimental setup and another setup in which the emission optics of a standard fluorescence microscope have been modified. Images of laser spots and of fluorescent beads were acquired using these setups to evaluate the performance of the imaging system when using dichroics of different flatness. Theoretical calculations and computer simulations were performed to specify required flatness of a dichroic mirror for different applications. The effectiveness of these results is also demonstrated by imaging cellular samples. It is expected that the findings in this research can be used to develop an industry standard for specifying the flatness requirements of dichroic mirrors for fluorescence microscopy applications.

2299/B676

A Perfusion-Enabled Cell and Specimen Compressor for Long-Duration Immobilization of Cells and Caenorhabditis elegans.

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A microcompressor is a high-precision mechanical device that flattens and immobilizes living cells and small organisms for optical microscopy. The process involving gentle flattening and immobilization has obvious benefits to allow for better visualization of sub-cellular structures and organelles. We have developed an advanced model based on the Aufderheide rotocompressor to view trapped specimens under Köhler illumination and with fluorescence microscopy. The older design, while engineered to delicately trap individual cells with micron precision, consisted of a closed chamber and made such devices unsuitable for holding live specimens over extended times. Furthermore, our newer devices can 1) incorporate a microfluidic perfusion system which allows the addition of nutrients, chemotactants, drugs, and other molecules, and/or (2) a PDMS platform that cushions the specimen to ease the compression stress and (3) can work on both inverted and upright microscopes. The new rotocompressors have been used to image the development of the neural network of immobilized C. elegans over the course of many hours. The usefulness of this device extends beyond C. elegans to many different specimen types and usages. for instance, we have immobilized and flattened individual Danio rerio embryos, Dictostelium discoideum cells, highly motile protozoa and bacteria, and mammalian cells.

2300/B677

Watching Cell Dynamics by Using a Combined Atomic Force - Optical Microscope System.
Keeping cells alive over a large period of time is a key requirement in microscopy imaging. We have developed a closed perfusion chamber that permits the simultaneous optical and AFM (Atomic Force Microscopy) observation of the sample, temperature control, maintenance of physiological incubation conditions, and perfusion of agents to trigger sample responses. That device was tested on different cell types in three experiments involving cell dynamics. Cells were spread on glass bottom Petri dishes that fit with the chamber and imaged by using Bright Field, DIC (Digital Interference Contrast), fluorescence and AFM. First tests were carried out on living fibroblasts. While constantly moving and establishing connections between each other, cells were successfully imaged in real-time by AFM over 24 hours. Second, the AFM tip was used as an indenter to induce a mechanical stress on fibroblasts kept alive over 48 hours. The response of the cell was tracked by fluorescence imaging, as the stress caused a brutal release of cations. Finally the perfusion chamber was used to scan co-cultured cells and observe their behavior and interactions in real-time over several days. Taken together, those results demonstrate the unique capability of our perfusion chamber and open the way to exciting discoveries in the field of cell culture and cell to cell communication.

2301/B678
Multiplexed Microfluidic Perfusion System for Live Cell Imaging.
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We have developed a microfluidic device that enables simultaneous imaging of live mammalian cells in up to 16 independent flow chambers on a 96 well plate. Each culture unit is fluidically isolated, with 3-6 upstream solution reservoirs to the 100 nanoliter cell chamber. perfusion rates between 1-10 microliters per hour are regulated by a set of high resistance flow barriers to ensure uniform, laminar solution flow to cells. The microfluidic plate is formatted to fit a standard 96 well plate to facilitate operation and handling. Reservoir wells are accessed using a standard pipet, and no fluidic connections are necessary to control flow. The cells adhere to the thin glass bottom surface of the plate, allowing for high magnification time-lapsed microscopy on any standard inverted microscope. The microincubation arrangement enables cells to be cultured for over 7 days without disturbing the microscope stage. A key benefit of the microfluidic culture system is the ability to easily control complex media exposure profiles to live cells. We demonstrate this capability with 3 example experiments. First, cells were alternately exposed to a drug solution and medium, switched periodically every 4 hours. Second, a ramp of drug concentration was applied to cells. Third, cells were exposed to a stable spatial concentration gradient of drug across the chamber. In all cases, cells were imaged via time-lapsed microscopy for morphological response. The major technological innovation of this work is to present a better tool for observing signal/response behaviors in live cells utilizing existing microscope equipment and assays. While most current perfusion systems rely on bulky and inefficient flow systems, our microfluidic method ensures the highest quality data while minimizing setup, cost, and microscope time. In addition, this method enables experiments not currently possible with other approaches (e.g. 16 flow experiments at once, stable gradient exposure, complex exposure patterns). This technology has potential impact in many applications including: gene induction/regulation studies, protein localization kinetics, Ca^{2+} flux assays, cell migration and motility, cell division mechanics, and apoptosis.

2302/B679
Microfluidic Devices for Long-Term Time Lapse Imaging.
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With the widespread use of genetically encodable fluorescent proteins such as GFP, live imaging is becoming increasingly important in cell and developmental biology. Such experiments allow insights into cell migration and morphology beyond that which can be gained from imaging fixed tissue. Time lapse experiments generate a new set of challenges, however, including how to maintain the health of a sample over the course of an experiment and how to track fluorescently labeled cells as they change position within a sample during normal development. To address these concerns, we design microfluidic devices to restrict the movement and optimize the culture conditions for several unique tissue types. These devices consist of small channels cast into polydimethyl siloxane (PDMS), a gas permeable, optically transparent, biologically compatible elastomer. Once cast, the devices are bonded to coverglass and the sample and media are introduced into the imaging chamber. The small scale of the channels allows for rapid exchange of culture media, and minimizes the total volume of media required for the experiments. To simplify cell tracking, we use a combination of commercially available software packages during acquisition and post-capture processing. Here we present examples of microdevices, their specific applications, and image analysis results from an array of model organisms.

2303/B680

High Resolution 5D In Vivo Cell Imaging in Humans Based on Multiphoton Tomography.

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Objective. Clinical non-invasive multiphoton tomography to obtain in-vivo optical biopsies with subcellular resolution based on two-photon autofluorescence and second harmonic generation. Methods. The multiphoton tomograph DermalInspectTM (JenLab) as novel 1M class medical system using a femtosecond-laser source which operates in the near-infrared spectral range has been employed. Non-linear excitation allowed to detect endogenous cellular fluorophores inside human skin such as NAD(P)H, flavins, melanin, keratin, and porphyrins down to a tissue depth of 200 µm without applying any contrast agents. In addition, the extracellular matrix components can be visualized. Spectral and fluorescence lifetime imaging have been performed by time-resolved single photon counting. Results. The multiphoton tomograph clearly visualized individual intratissue cells, intracellular mitochondria, melanosomes, and the morphology of the nuclei as well as extracellular matrix elements. Individual melanocytes, distorted cell morphology and large cell-cell distances were clearly imaged in the upper epidermis of patients with malignant melanoma. So far, more than 500 patients and volunteers in Europe, Asia, and Australia have been investigated with this cell imaging tool for the early detection of skin cancer and the morphology of In Vivo skin. Conclusion. In Vivo multiphoton tomography enables for the first time non-destructive high-resolution morphological and functional imaging of cells and intracellular structures in human skin without the need of exogenous markers based on endogenous fluorophores.

2304/B681

Three Electrode Control of the NanoDeposition of Gold Nanoparticles with Atomic Force Controlled Capillary Electrophoresis.

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Controlled deposition of metallic features such as nanoparticles with high spatial accuracy has great interest for different applications including surface plasmons, surface enhanced Raman scattering (SERS), nanophotonics and nano biophysics. Lithography based SPM techniques have been shown as potential methodology for accurate, localized deposition of material at nanometer scale. We report an accurate deposition of high resolution features of single gold
nanoparticles using three electrodes and AFM controlled capillary-based fountain pen nanolithography. Three electrodes are attached, one on outside of the metal coated glass probe, second on inside of the hollow probe in solution contained in the capillary, and third electrode on writing surface. The three electrodes provide electrical pulses for accurate control of the deposition and retraction of the liquid from the surface. We will demonstrate depositing of single gold nanoparticle with size of 1.2nm onto surfaces such as semiconductors.

2305/B682
The New Atmospheric Scanning Electron Microscope Enables the Observation of Cells in Buffer Under Normal Atmospheric Pressure.
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Optical microscopy, especially with fluorescence labeling, is a powerful tool for the dynamic observation of cells, but has limited resolution (200 nm). The Scanning Electron Microscope (SEM) has higher resolution, but the sample must be observed in vacuum. A capsule system sealed with a polyimide film window has been developed, where cells are cultured inside the capsule and the capsule is placed in the SEM, but it has a capacity of only 15 μl and does not allow for long culture periods or easy administration of drugs. To address these limitations, we have developed the Atmospheric SEM (ASEM) with an open culture system. The SEM has been inverted so that the electron gun is below an open ASEM dish, which has a silicon nitride (SiN) window built into its base. The SiN film window is 100 nm thick, allowing electron transmission but sustaining a 1-atm pressure differential. The thickness of SiN film can be reduced to 10 nm. The detachable ASEM dish has a several-ml capacity, allowing prolonged culture in a CO2 incubator. An optical microscope (OM) is positioned above the open culture dish for quasi-simultaneous observation. This system eliminates time-consuming pretreatment, especially dehydration; cells need only be fixed. Speed, open configuration, and high resolution (8nm) define this system. As an example, COS7 cells cultured on an ASEM dish were fixed and dyed, requiring about one minute. The Endoplasmic Reticulum (ER) was identified with the OM, while the ASEM, at x20,000 magnification, displayed amazingly fine connections within the ER. The ASEM also revealed the localization of tubulin and F-actin in pyramidal neurons using its OM, and their fine structures were further revealed by its SEM. The ASEM allows the effects of drugs on cells to be observed using the OM, while at a definitive moment the cells can be fixed for immediate ASEM observation. The ASEM can also be a high-throughput immuno-electron-optical microscope, using fluorescence/gold labeling of proteins. Detailed studies of sensitive epitopes at high resolution will open doors for the research of various proteins important in cell biology.

2306/B683
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Optical microscopy with fluorescent labeling is a powerful tool for cell biology, but has limited resolution (200 nm) due to the wavelength of photons. The Scanning Electron Microscope (SEM) produces higher-resolution images, but the sample must be observed in vacuum, requiring pretreatment for preservation, e.g., dehydration and drying, which involve the possibility of deformation. To overcome these difficulties, a capsule system sealed with a polyimide film window has been developed, where cells are cultured inside a capsule and the capsule is placed in the SEM. This, however, has a capacity of only 15 μl and does not allow for long culture periods or easy administration of drugs. To address these limitations, we have developed a combination microscope of Atmospheric SEM (ASEM) and Optical Microscope (OM), having an open culture system. The SEM has been inverted so that the electron gun is below an open

- 1111 -
ASEM dish, which has a silicon nitride (SiN) window built into its base. The SiN film window is 10 - 100 nm in thickness, allowing electron transmission but sustaining a 1-atm pressure differential. The ASEM dish has a several-ml capacity, allowing prolonged culture. Since dehydration is not necessary, ASEM drastically shortens the pretreatment period. Cells are treated only with a fixing reagent. In this system, an optical microscope (OM) is positioned above the open culture dish for quasi-simultaneous observation with the ASEM. This enables correlative microscopy of photons and electrons, and, with fluorescence labeled microscopy, enables the identification of fine shapes revealed by the ASEM. ASEM also allows dynamic observation of the effects of drugs on cells using the optical microscope, while at a definitive moment the cells can be fixed for immediate ASEM observation. Speed, open configuration, and high resolution (8nm) define this system. In this presentation, examples of such observations are presented, and the limit of observable depth is also discussed.

2307/B684
The Atmospheric SEM Observes Nuclei in the Surface Level of Tissue Without Thin-Sectioning.
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The Atmospheric scanning electron microscope (ASEM) observes cells and tissues in open medium through SiN film. Using the ASEM, subcellular structures including DNA and/or RNA were very strongly stained and visualized above the dark background. Nuclei, including chromatin and nuclei, and chromosomes, were particularly emphasized during these observations. The most important indicator for intra-operative cancer diagnosis is the size of the nucleus, which has until now been determined using OM. However, this method takes at least 15-30 minutes, during which period tumor excision surgery is frequently suspended. Among the diagnostic procedures, cryo-thin-sectioning of tissues is the most difficult and time consuming. Since the ASEM scans only the surface, the surface level of a section of tissue can be observed without thin-sectioning. In tests of the ASEM, a half-brain surgically resected from a goldfish was fixed and stained, then observed using the ASEM. The nuclei in the surface level of the tissue were brightly distinguished above the dark backgrounds, and neurite-like fibers were also stained, though more weakly. The ASEM, in combination with nucleotide specific labeling, further raises the possibility of high resolution karyotyping, which will aid in the diagnosis of slight chromosomal deviations closely related to genetic diseases, until now difficult to identify. Another opportunity for the diagnostic use of the ASEM is the identification of pathogens. Due to its high resolution capabilities, the ASEM can visualize the details of various bacteria, including the lactobacillus and photosynthesis bacteria. The ASEM does not require the time-consuming pretreatment usual for electron microscopy, just fixation. It is widely applicable not only to basic biology but also to cancer diagnosis, pathogen diagnosis, drug discovery, and food science.

Epithelia II (2308 – 2322)

2308/B685
Enhanced Antioxidant and Prevent Prothrombotic Effects by Substance P Scavenger on Rat Lung after Acute Exposure To Oil Smoke.
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Objective: Airborne particulate matter, from cooking oil, smoking, engine exhaust and other sources, is associated with the development of atherosclerosis and myocardial infarction. In order to explore the cellular and molecular events following exposure of rats to lard oil smoke, we measured the generation of reactive oxygen species (ROS), substance P, cellular adhesion molecules, and thrombosis in relation to inhibitors of substance P, the NK-1 receptor, and antioxidants. Methods: Rats were exposed to oil smoke for 120 min with or without 20 min
pretreatment with lovastatin (substance P scavenger), L733060 (NK-1 receptor antagonist), vitamin E (antioxidant) or catechins (antioxidant). The levels of substance P and ROS were measured. Histological studies observed ROS damage in the form of HEL adducts. The prothrombotic effects of oil smoke exposure were measured by experimental induction of thrombosis in vivo. Results: Both substance P and plasma ROS were significantly increased in oil smoke exposure (OSE) than in control group. Histologically, OSE caused microscopic hemorrhaging and the sloughing of cells around the bronchi of rat lung; at the same time, the amount of inflammatory cells observed in the lung tissues were increased compared to normal rat lungs. OSE significantly increased ROS damage (HEL adduct levels) and the size of experimentally induced thrombi. Pretreatment significantly decreased the HEL/β-actin ratio following 120 min OSE: 0.54, 1 μg/kg L733060; 0.65, 4 mg/kg lovastatin; 0.52, 2.5 mg/kg vitamin E; 0.43, 2.5 mg/kg catechins. The pretreatments statistically significantly reduced all of these effects of OSE. Conclusions: We established a connection between oil smoke exposure and thrombosis which involves substance P and its receptor, the NK-1 receptor, and ROS. This study helps establish a mechanistic explanation of how airborne particulate matter can increase the risk of cardiovascular illness.

2309/B686
Development of an In Vitro Alveolo-Capillary Model Using Rat Alveolar Type I and Microvascular Endothelial Cells.
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The alveolo-capillary membrane (ACM), composed of alveolar epithelial and microvascular endothelial cells (MVECs), plays a vital role in physiologic functions such as gas, ion and water exchange. In Vitro models are often used to gain insight into the mechanisms and dysfunctions of the ACM. This has led to the use of alveolar type I (AT I)-like cells derived from alveolar type II cells, because problems with the isolation and propagation of primary AT I cells have limited the development of a co-culture model to study the ACM. The purpose here was to develop a co-culture model composed of primary AT I cells and MVECs that could be used for future studies. AT I cells and MVECs were isolated from the lungs of 3 month old male Fischer 344 rats. To characterize the primary AT I cells we measured: (a) presence of AT I specific markers T1α and aquaporin-5 (AQ-5); (b) morphological characteristics (area, perimeter, population density); (c) growth characteristics; and (d) percentage of apoptotic cells and perinuclear mitochondrial location. Purity of AT I cells was determined by the presence of T1α, AQ-5, and the absence of VE-cadherin. Purity of MVECs was determined by the presence of VE-cadherin and von Willebrand factor. Apoptosis was determined by staining for mitochondrial membrane integrity; this also permitted visualization of mitochondrial location. The results showed a highly pure preparation of primary AT I (96%) and MVECs (98%) with a yield of 0.47 to 0.94 million AT I cells per rat. Further, AT I cells were propagated through population doubling 114 with <1% apoptotic cells. To produce a co-culture model, primary AT I cells were seeded on the top surface and MVECs on the bottom of a Transwell Fluroblok filter. Fluorescent labeling of each cell type showed no transmigration through the 1 micron pores, and cells in co-culture remained viable for over 14 days. In conclusion, we report the successful development of a co-culture Transwell model system using primary AT I cells and MVECs that may be used to gain insight into the physiological functions and dysfunctions of the ACM.

2310/B687
Baobab Is a Negative Regulator of CFTR-Dependent Fluid Secretion.
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To better understand how CFTR functions in vivo, numerous studies have searched for and identified potential interacting partners but to date, there is little evidence linking any of the known CFTR interactors to its activity. During a forward genetic screen in zebrafish, aiming to identify genes regulating endodermal organ development, we identified a mutant, which we named baobab (bao), after the African tree that accumulates water during the wet season, which exhibits a dramatically enlarged fluid-filled gut tube. This phenotype is accompanied by a stretching of the gut epithelium and a loss of microvilli. Most interestingly, we have shown that various CFTR inhibitors significantly reduce the appearance of enlarged guts in bao mutant embryos. Conversely, pharmacological activation of the CFTR channel leads to an expansion of the gut lumen that closely resembles that of bao mutant embryos. We have positionally cloned the bao locus. Bao is a soluble protein expressed in the gut and other endoderm-derived organs. Loss of function in baos866 or knockdown using anti-sense morpholinos leads to fluid accumulation in the gut but does not affect CFTR expression or localization, suggesting that the phenotype results from the uncontrolled activation of the CFTR channel. Moreover, overexpression of bao in MDCK cells abrogates the stimulatory effect of cAMP in CFTR-dependent fluid secretion. Furthermore, depletion of Bao leads to the accumulation of fluid in MDCK cysts that is reminiscent of the phenotype observed in vivo in zebrafish. Co-immunoprecipitation experiments showed that CFTR interacts with WT bao but not with the mutant version of protein. Altogether our results indicate that Bao is a new negative regulator of CFTR activity and that control of fluid secretion is crucial for organogenesis. This work establishes a new genetic model system for studying the regulation of the CFTR channel that will contribute to the understanding of the pathophysiology of Cystic Fibrosis (CF) and several intestinal secretory conditions.

2311/B688
Role of Epithelial Intestinal PTEN Phosphatase in Enteroendocrine Cell Specification.
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Glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) are 2 hormones form the incretins family secreted by the K and L endocrine cells respectively. These hormones, produced by the intestine, are known to affect the proliferation, survival and secretion of pancreatic β cells. Recently, we have demonstrated that intestinal epithelial Pten impact on the cytodifferentiation of the secretive cell lineage. However, the role of Pten on the various endocrine sub-populations has not yet been explored. AIM: to investigate the role of Pten in the specification of the enteroendocrine sub-population as well as its potential to impact glucose metabolism. METHODS AND RESULTS: With the use of the Cre/loxP system, we have generated a mouse with the Pten gene deleted exclusively in the intestinal epithelium. We have analyzed whether loss of epithelial Pten alters the specification of the various endocrine sub-populations. As shown previously, chromogranin a (CgA) immunostaining revealed a decrease in the number of labelled cells in the mutant animals. However, CgA is not expressed by the endocrine sub-populations D, I, K and L. Our analysis revealed an increase in I, K and L cell sub-populations as demonstrated by an increase in the numbers of cells labelled for CCK (I), Gip (K) and GLP (L). This was confirmed by qPCR analysis increases in mRNA levels for CCK (1.7x), Gip (1.7x) and GLP-1 (2.6x). Thus, we investigated the impact of the loss of intestinal Pten on glucose metabolism. An oral glucose tolerance test revealed that mice with impaired Pten have accelerated glucose absorption as well as increased glucose metabolism. qPCR analysis on intestinal mucosa showed that mRNA levels of the glucose transporter Sglt-1 were increased in mutant animals. CONCLUSION: Our results revealed that loss of epithelial Pten affects endocrine sub-population specification. Interestingly, we showed that Pten deregulation in the intestinal epithelium impacts on glucose.

2312/B689
TNF-α-mediated Inhibition of Hsp70 Chaperoning Activity Results In Down-Regulation of Atypical PKCι in Intestinal Cells.
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TNFα is one of the most recognized proinflammatory mediators involved in the pathogenesis of Inflammatory Bowel Disease (IBD). In the present work we investigated the mechanisms underlying TNFα-induced signaling in CACO-2 cells (a colon carcinoma cell line which expresses many features typical of human enterocytes). Treatment of CACO-2 cells with TNFα in concentrations which are within the range observed in human chronic colitis, resulted in a steep decrease in the amount of atypical PKCι protein. PKCι has a well-established role in the onset and development of tight junctions and is known to be the one of the key organizers of cell polarity. It was shown before, that TNFα affects tight junction permeability through the activation of NF-κB signaling pathway. However, our RT-qPCR experiments did not reveal any difference in PKCι mRNA levels between control and TNFα pre-incubated cells, indicating that the signaling events responsible for the decreased amount of PKCι are post-transcriptional. Interestingly, we also did not observe any changes in the activity of PDK-1, the upstream kinase, which provides the priming phosphorylation and is essential for PKCι function. On the other hand, incubation with proteosomal inhibitor MG-132 resulted in the accumulation of significantly higher ubiquitinylated PKCι protein in TNFα treated cells, allowing us to conclude that TNFα promotes proteosomal degradation of PKCι. Recently, we reported the novel mechanism of PKCι rescue and re-phosphorylation, which involved the intermediate filaments (IFs) and Hsp70. Interaction of PKCι with Hsp70 and IFs is crucial to prevent an activity-induced PKCι degradation and regulates the signaling half-life of this kinase. Using an In Vitro reconstruction assay we found that TNFα treatment almost completely abolished PKCι re-phosphorylation. Moreover, Hsp70 from TNFα treated cells exhibited impaired chaperoning activity in luciferase refolding experiments. Altogether, we showed that TNFα affects the post-translational mechanism, which controls the steady state levels of PKCι and, thus, tight-junction permeability.

2313/B690
Crohn’s Disease Associated Protein Dlg5/Lp-Dlg Controls Epithelial Mesenchymal Transition in LLcPK1.
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Dlg5 is a member of MAGUK (membrane associated guanylate kinese) adaptor family proteins. Dlg5 localizes at cell-cell adhesion sites and associates with β-catenin and vinexin. Dlg5 has been reported to be a susceptibility gene for Crohn’s disease, one of the two major forms of inflammatory bowel disease. However, the physiological function of Dlg5 is not still understood. Epithelial cells can convert into mesenchymal cells by a process called epithelial to mesenchymal transition (EMT). Epithelial cells have robust cell-cell adhesions mediated by specialized structure. In contrast, mesenchymal cells have higher motile activity and robust cell-ECM (extracellular matrix) adhesion. The process of EMT is well established to play important roles in various physiological and pathophysiological conditions, including embryogenesis and renal fibrosis. In this study, we investigate the roles of Dlg5 in LLc-PK1, renal epithelial cells, in which Dlg5 expresses at high level. We found that TGF-β treatment induced EMT and decreased Dlg5 expression in LLc-PK1. Dlg5 knockdown by siRNA induced the SMA (α-smooth muscle actin) expression, a mesenchymal marker, and decreased the E-cadherin expression, an epithelial marker. Dlg5 re-expression cells suppressed Dlg5 Knockdown induced SMA expression. Quantitative RT-PCR revealed that the Dlg5 knockdown induced increase of SMA mRNA and decrease of E-cadherin mRNA suggesting that Dlg5 attenuates an EMT inducing signal. We next analyzed the effect of Dlg5 knockdown on the TGF-β-induced signals. Smad2 phosphorylation was not affected by Dlg5 knockdown, but p38 phosphorylation was promoted by Dlg5 knockdown. Inhibition of TGF-β signal by TGF-β receptor inhibitor or transfection of smad7 suppressed SMA up-regulation and E-cadherin down-regulation in Dlg5 Knockdown cells. These results indicate that Dlg5 regulates EMT mediated by TGF-β receptor, in LLc-PK1 cells.
2314/B691
A New Role for Adenomatous Polyposis Coli (APC) in Controlling the Polarity of Epithelial Cell Extrusion: A Possible Link to Colon Cancer Invasiveness.
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We defined a mechanism for how an epithelium copes with dying cells while maintaining a functional barrier, termed ‘extrusion’. Here, the apoptotic cell signals its neighbors to form and contract an intercellular actin/myosin II ring, which squeezes circumferentially and downward to push the dying cell out of the layer. Importantly, we have found that extrusion is not limited to dying cells, since many inhibitors of apoptosis do not block extrusion. Thus, tumor cells, typically blocked in the apoptotic pathway, could use extrusion to exit the epithelium and initiate metastasis if they exit basally rather than apically. Most apoptotic cells we have observed extrude apically, however, monolayers deficient for the APC tumour suppressor gene (Adenomatous Polyposis Coli) extrude predominantly basally (75 ± 5% compared to 19 ± 2% in wildtype lines). In wild type cell lines, microtubules reorganize towards the dying cell to target actin and myosin contraction basolaterally, thereby driving extrusion apically. However, colon cancer cell lines with APC mutations do not reorient microtubules or target actin/myosin basolaterally so that cells cannot extrude apically. Microtubule disorganization is likely due to a commonly occurring truncation (in greater than 80% of colon tumors) that deletes the microtubule-binding domain of APC, preventing it from linking microtubules to the cortical actin at the extruding ring. APC localizes to the extrusion ring throughout the extrusion process even in the presence of nocodazole, suggesting that APC recruitment to the actin cortex is upstream of microtubule reorientation. Because APC mutations increase basal extrusion rates, we propose that additional, frequently occurring mutations that block apoptosis (such as in RAS) could drive increased tumor cell invasiveness.

2315/B692
Role of Drebrin in Apical Membrane Organization and Epithelial Morphogenesis.
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During epithelial morphogenesis, some epithelial cells switch from a cuboidal to a columnar shape developing a sub-apical terminal web and a brush border made of densely packed microvilli. We have studied the role of Drebrin, an actin-binding protein involved in the remodelling of actin cytoskeleton, during the process of epithelial morphogenesis. We observed that Drebrin in human intestine localized at the sub-apical region closed to the terminal web and along the lateral membrane. The same localization was observed in human colonic Caco2 epithelial cells, validating our cell culture model. We used transient transfection of siRNA to down regulate its expression in Caco2 cells. Depletion of Drebrin expression strongly affected epithelial morphology; knockdown cells were unable to acquire a columnar shape and to form a tightly packed monolayer of cells while neither apico-basal polarity nor tight junctions were affected. Interestingly, scanning electron microscopy observations clearly showed a disorganisation of the brush border in cells knockdown for Drebrin accompanied by a weaker expression of DPP IV and Sucrase Isomaltase, two markers of intestinal apical differentiation. Furthermore, the terminal web that is a key component of brush border organization does not form in the Drebrin knockdown cells as actin and fodrin accumulation in the sub-apical domain was prevented. In the absence of Drebrin, Myosin II organization at the apical pole was also affected, suggesting that Drebrin is important for the stability of the actin-interacting proteins that form the terminal web. Our hypothesis is that Drebrin is necessary for actin re-organization during apical differentiation by allowing the formation of a branched actin terminal web allowing the anchoring of the brush border. In addition the lack of connection between Myosin II and the actin network at the apical belt of junctions prevents apical constriction necessary for a densely packed epithelial layer.
Estrogen exposure during the early post-natal period affects male growth, physiology, and susceptibility to disease in adult life. High dose estrogen in the early postnatal life causes the prostate gland to show a reduced expression of the androgen receptor and inability to respond to androgen stimulus. In this context, we decided to study key signaling regulators of ventral prostate functioning after early postnatal exposure to high-dose estrogen, by applying morphological, histochemical, biochemical and molecular analyses. Our results showed that luminal epithelial cells in the estrogenized prostate were shorter, less polarized, and had smaller nuclei containing more-compacted chromatin, suggesting that a general mechanism of regulating gene expression and protein synthesis could be installed in epithelium of the estrogenized VP. In order to explore this hypothesis, we analyzed nucleolar morphology, and measured the amount of RNA and the level of methylation of the 45S ribosomal RNA promoter region. These data indicated that the nucleolus was dismantled, the methylation at the 45S promoter was increased (~5-fold), and that the absolute amount of 18S rRNA was reduced to lesser than 1% of the control. We also found a decrease of mTOR phosphorylation and its direct downstream target 4EBP. It is known that mTOR-induced signaling is a pivotal pathway of cell metabolism, which is able to control gene transcription and protein synthesis. Taken together, the results obtained here support the idea that the estrogenized prostate maintains a very low transcriptional level and protein turnover by affecting canonical signaling pathways and promoting nuclear and nucleolar changes. Funding: FAPESP/CNPq

Cadmium (Cd) is a wide spread environmental pollutant, characterized by its toxicity in various human and animal organs. Although it is well known that cadmium is associated with adverse effects on male reproductive organs of rats, very few studies have quantified the morphological alterations caused by this metal. Male Wistar rats were randomly assigned to three groups of 12 animals sacrificed after 7 and 56 days (d). Cadmium chloride solution was injected i.p. as a single dose of 1 or 1.2 mg/Kg BW. Testis and epididymis alterations were quantified by morphometric techniques. The lower dose did not cause marked alterations in morphometrical and morphological analyses. The higher dose caused severe and significant alterations. The metal caused significant reductions of testis and epididymis weights (7 and 56 d) and of the seminal vesicle weight (7 d). However, the ventral prostate, seminal vesicle and coagulating gland weights did not alter in any group. A significant reduction was observed in the gonadossomatic index and in the length of seminiferous tubule (ST) after 7 and 56 d. Cd significantly reduced the ST diameter after 56 days. The decrease in the volume density of ST was accompanied by an increase in the interstitium volume density (7 and 56 d). After 56 d, a significant decrease in the tubular compartment occurred in epididymis caput and cauda. After 7 d, tubular lumens were filled with degenerated germ cells and multinucleated spermatid aggregates, with apoptotic nuclei. After 56 d, the ST was only lined by Sertoli cells. Scanning electron microscopic observation of the interstitial tissue of control animals, after 56 d, showed in an intricate and delicate three-dimensional lattice with large fenestrae encircling each ST. The seminiferous epithelium was intact and the ST lumens filled with spermatozoa. In the Cd group, the interstitial tissue was very compact, with few fenestrae. The ST showed reduced epithelium height and
absence of spermatozoa in the lumen. The results demonstrate that a very small difference in the Cd dose administered causes a sudden increase in testicular damage, apparently overcoming the natural defenses of this tissue.

2318/B695

Expression of Luteinizing Hormone (LH) Subunits in Rat Spermatozoa.
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The production of luteinizing hormone (LH) and its biological action have been considered to be only occurred in pituitary and gonads, respectively. However, several lines of evidence indicate the presence of LH/hCG receptor in nongonadal but some reproduction-associated tissues such as female reproductive tracts of rodents, human and other species. Furthermore, the evidence for the extrapituitary production of LH in rat gonad suggests the novel function of the hormone as a local factor. The present study was carried out to elucidate whether rat spermatozoa express LH subunit genes. In the reverse transcription-polymerase chain reaction (RT-PCR), the target cDNA fragments for LH beta subunit and common alpha subunit for the pituitary glycoprotein hormones were precisely amplified from the rat epididymal spermatozoa. Fluorescent immunohistochemistry with specific antisera against the subunits revealed that positive stainings were intensively found in midpiece of spermatozoa. The spermatozoa which were pre-incubated with antiserum against beta subunit could bind to acrylic beads coated with protein-A, suggesting the rat spermatozoa's LH beta subunit might be functional. In conclusion, the present study provides evidence that genes for LH subunits are expressed in rat spermatozoa. With the previous studies, these findings suggest that the locally produced LH could play some role(s) such as regulation of gamete transport and fluid secretion in the female reproductive tracts.

2319/B696

Organization and Bacterial Assemblage of the Glandular Papillae of the Comb Jelly Mnemiopsis leidyi.
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Mnemiopsis leidyi (Phylum Ctenophora: Class Lobata) is a gelatinous metazoan endemic to coastal Gulf of Mexico and Eastern United States. It is a notoriously invasive animal, commonly undergoing transatlantic transport via ship ballast water. M. leidyi secretes vast quantities of mucus, possibly in an attempt to sweep the surface free of contaminating microbes; as a result it appears to be populated by a very limited An intriguing feature of its anatomy is a collection of wart like glandular papillae, found in and just below the epithelium. Papillae are much more common on large, presumably older individuals, which may have hundreds of papillae. Papillae tend to be concentrated on the aboral epithelium, and on the outermost surface of the proximal-lateral aspects of the lobes, although in very large individuals they may be present on the entire outer surface epithelium. Virtually nothing is known about the papillae, although it is thought that they secrete mucus. We show here that papillae are a distinct organ system, being consistently composed of several discrete components, which have distinct functions. Papillae are supported by a basketlike arrangement of smooth muscle fibers (as revealed by Alexa 488 phalloidin staining), which underlie 1 to 12 large central gland cells that resemble goblet cells, each of which bears a colored oil droplet. Hoechst staining reveals the basal location of the gland cell nuclei, and the presence of numerous bacteria. Noxious stimulation of papillae results in the contraction of the muscular basket, eversion of the gland cells, and release of mucus. A 16S rDNA analysis of papillar bacterial assemblages indicates a wide diversity of bacterial taxa relative to the bacterial diversity of surrounding water or the gut. Scanning electron microscopy will be used to display visual documentation of bacteria associated with the papillae and ctenophore along with Transmission Electron Microscopy to observe the ultra structure of the papillae. Support: NSF AL-EPSCoR EPS 0447675, AU-Cell and Mol. Biology Program scholarship to EWD, and NSF MCB-0348327 to AGM
Delicate coastal marine jellies are repeatedly exposed to dangerous epithelial wounds from predation and interaction with coastal obstacles. The lobate ctenophore Mnemiopsis leidyi is capable of undergoing rapid scar-free epithelial wound repair. A wound measuring approximately 3 mm long heals in as little as 30 minutes with no formation of a scar. Our lab seeks to develop the ctenophore as a model system to study the cellular and molecular mechanisms underlying rapid wound repair. Minutes after wounding, thousands of 10-15 μm diameter cells migrate up into the wound site, where they aggregate and send out processes that reach across the wound gap (Dodson/Moss, 2007 ASCB). Ctenophores are also capable of regenerating excised portions of their canals (Dodson/Moss, 2007 ASCB). Light microscopy has revealed how the wound cells interact both In Vivo and in vitro, as well as how the muscle fibers surrounding the wound reorient in a purse string like arrangement. Alexa phalloidin staining analyzed with Laser Scanning Confocal Microscopy, has revealed a major role of actin at the wound site. Using RT-PCR we have been able to identify an initial array of genes involved in the wound cascade. When wounded the ctenophore rapidly initiates the wound response. Muscle fibers surrounding the wound immediately reorient and serve as a scaffold for the wound cells migrating into the wound site. Epithelial cells begin migrating toward the wound, forming an aggregate of cells that becomes the new epithelium. The cells attached to the muscle fibers underneath the damaged epithelium, stretch across the wound gap and actively contract to bring the wound edges closer together. Numerous cytoskeletal components including Ankyrin, Arp 2/3, and Actin Depolymerizing Factor, as well as other wound response components such as Wnt, Hsp 60 and 90, and TGF- beta have all been shown to be involved in ctenophore healing. Understanding the mechanisms responsible for the rapid wound repair in ctenophores can help correlate this mode of healing with wound repair in higher organisms, creating a possible avenue of biomedical research in humans. Support: NSF AL-EPSCoR EPS 0447675, AU-CMB scholarship to MD, NSF MCB-0348327 to AGM.

During a morphological survey on leaves of Brazilian Cerrado species from Amaranthaceae family, we found a unicellular parasite on leaves from Gomphrena hermogenesii J.C. de Siqueira and Pfaffia gnaphaloides (L.f.) Mart. (Amaranthaceae) Leaves. S. F. Carvalho, J. R. Correa, S. N. Bão; Biologia Celular, Universidade de Brasília, Brasília, Brazil.

During a morphological survey on leaves of Brazilian Cerrado species from Amaranthaceae family, we found a unicellular parasite on leaves from Gomphrena hermogenesii J.C. de Siqueira and Pfaffia gnaphaloides (L.f.) Mart. In Brazil, this family is important by its use as medicinal and food plants. This work aims to describe the parasite morphological aspects and its relation with its hosts. Leaves from 2-6 specimens of Gomphrena hermogenesii J.C. de Siqueira and Pfaffia gnaphaloides (L.f.) Mart. were collected at vegetative and/or flowering stages. The middle portions of the leaves were fixed in 2% of glutaraldehyde, post-fixed in 2% osmium tetroxide, followed by in block staining. The samples were dehydrated in acetone and embedded in Spurr epoxy resin. Ultra-thin sections were obtained and analyzed under TEM JEOL JEM 1011. Tissue sample from young mature leaves (20-25 days old), collected during flowering stage of both species, shows unicellular organisms and morphological cells alterations which suggests an infection process. In the intercellular spaces, particularly in the spongy parenchyma tissue, were observed small organisms compatible in size with bacteria. Few of these organisms were found in the xylem elements of xylem, exclusively in P. gnaphaloides leaves. Inside the cells of leaf tissues from both plant species were found an organism with 4-16 μm length. These organisms show cell wall, mitochondria and membranous organelles that match morphologically with nucleus and endoplasmic reticulum. These microorganisms are found associated with disrupted chloroplasts.
mitochondria and nucleus of its host cells. Bundle sheath chloroplasts of \textit{G. hermogenesii} on both samples show lipid droplets with 0.19-0.85 $\mu$m of diameter on non infected leaves and about of 0.05-0.11 $\mu$m on infected leaves. In \textit{G. hermogenesii}, a C4 species, these organisms seem to have more affinity with cells involved on energy producing. Our findings suggest lipids consumption and morphological cells alterations linked to these microorganisms. Further studies are ongoing in our laboratory in order to characterize these microorganisms and to establish if there is poisoning risk by the human leaves consumption of infected plants.

2322/B699
\textbf{Ricin and RCA-I Binding to Buccal Cells and Salivary Fluid.}
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The extremely toxic plant protein ricin is subject to biological and chemical weapons bans and is of concern as a tool of terrorists. There is no cure for ricin poisoning. Gaps exist in knowledge of the specific molecular identities of cell surface ricin-binding proteins. Buccal cells and salivary fluid are biological materials that could be exposed to ricin during poisoning. Study objectives were to assess if binding of ricin and RCA-I could be detected to buccal cell surfaces, salivary and buccal proteins and to determine molecular masses of ricin ligands. Salivary fluid and buccal cell fractions were isolated by centrifugation. Ricin and RCA-I-binding proteins were detected by lectin blotting after SDS gel electrophoresis of saliva and buccal cell proteins and also measured by enzyme-linked microtiter plate binding assays. Fluorescence microscopy with biotinylated ricin and RCA-I was used to visualize localization of ricin and RCA-I binding to buccal cell surfaces. After electrophoresis, lectin blots identified a 170kDa buccal cell protein band in reduced samples that bound to ricin, binding was absent or decreased in non-reduced samples. Major ricin-binding proteins in salivary fluid included 170-150kDa, 75kDa, 50kDa, 40kDa and 25kDA molecules. \textit{Clostridium perfringens} neuraminidase increased the binding of ricin to blots of salivary fluid proteins but had less effect on the binding of RCA-I. \textit{Vibrio cholerae} neuraminidase did not affect the binding of ricin and RCA-I to buccal cell proteins blots. In fluorescence microscopy and microtiter plate binding, ricin bound weakly to buccal cells in contrast to strong binding seen with RCA-I. Specific ricin and RCA-I -binding salivary and buccal cell proteins were detected by lectin blotting especially a common 170kDa protein. There were differences in the reactivity patterns of the related lectins RCA-I and ricin with buccal cells and saliva, even though both are reported to bind to galactose-terminated glycoconjugates. Binding to buccal cells and salivary proteins could be relevant to the bioavailability of ricin or dose reaching other tissues in the event of poisoning by the oral route.

\textbf{Leukocytes (2323 – 2334)}

2323/B700
\textbf{The Role of NADPH Oxidase in MAP Kinase Cascades Activation at Phagocytosis.}
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The macrophage ability to engulf pathogens into specialized endocytic vacuoles and to destruct them is crucial for a proper immune response. for a long time the main mechanism of intraphagosomal pathogen destruction was considered as a result of an oxidative damage of pathogen cells by reactive oxygen species (ROS) produced by NADPH oxidase (NOX). The latest data provided a strong evidence that the pathogen destruction is a result of electrogenic NOX activity that makes an intraphagosomal environment optimal for phagocytic proteases activation. at the same time NOX family proteins are expressed in most cell types where they regulate various signal transduction pathways. Particularly, H2O2 produced by NOX oxidize cysteins in a catalytic cleft of protein tyrosine phosphates (PTPs) allowing tyrosine kinases to phosphorylate downstream targets like MAPKs. However, a correlation between the NOX activation in
phagocytes and MAPKs activity remains unclear as well as a real time profile of H2O2 generation by a single phagocyting cell. The aim of this research was to explore a real-time H2O2 production and the main MAPK cascades activation in phagocyting RAW264.7 murine macrophages. Confocal ratiometric time-lapse imaging of H2O2 production in RAW264.7 phagocyting cells expressing HyPer, a genetically encoded probe for H2O2, revealed a transient wave of NOX activation starting immediately after serum opsonized zymosan uptake and lasting for 15-20 min. To understand correlation between the H2O2 production and the Erk1/2 and p38 MAPKs activities, we used immunofluorescent staining and western blot analysis with specific antibodies to phosphorylated Erk1/2 and p38 forms. We observed both Erk1/2 and p38 pathways activation in phagocyting RAW 264.7 cells. Erk1/2 but not p38 pathway activation requires NOX driven H2O2 production. Using a panel of inhibitors we demonstrated reciprocal p38 and Erk1/2 cascades inhibition in macrophages. Taken together, we revealed that the NOX function in phagocytes is nearly to be similar to that one in non-phagocyting cell types. H2O2 produced by NOX is extensively used for the modulation of the signal transduction pathways by phagocytes.

2324/B701
Insulin to the Rescue: A New Role in Macrophages.
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Insulin has been used in the past to treat wounds with a positive outcome, but not much was learned about the cellular and molecular mechanisms of insulin action in healing. Recently, we have shown that insulin application to acute wounds accelerates wound closure, stimulates keratinocyte motility, increases expression of cell adhesion molecules in the ECM and in keratinocytes, stimulates angiogenesis and improves dermal regeneration with reduced scarring (Liu et al., 2008; Liu et al., 2009). Therefore, we hypothesized that insulin affects monocyte/macrophage biology in the wound tissue leading to a decreased or modified inflammatory response. Using ELISA and western blot analysis, we show that insulin levels decline initially after wounding for 48 hours and then increase to a peak at 5 days before returning to basal levels whereas the insulin receptor levels increase through five days, decline through day 9, and then increase again upon wound closure around day 14. We also show that low dose insulin (10-7M) increases proliferation of THP-1 monocytes whereas high dose (10-6M) prevents proliferation, indicating that the drop in insulin in the wound earlier on is beneficial to allow monocytes to proliferate before they differentiate into macrophages. However, after macrophages are well established in the tissue, the higher levels of insulin will prevent monocytes from proliferating and hence macrophages from building in the tissue. In addition, we found, using chemotaxis assays, that the higher levels of insulin promote macrophage migration. These results taken together provide evidence that insulin may be interacting with monocytes and macrophages during the inflammatory phase of healing to affect their behavior. We are currently focusing on deciphering the mechanisms by which insulin signaling exerts its effects on the wound healing process in both normal and diabetic wounds.

2325/B702
LIGHT/TNFSF14-Deficient Mouse as a Potential Model to Study Impaired Resolution of Inflammation during Wound Healing.
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Acute wound healing progresses through a series of overlapping phases, involving inflammation, granulation tissue formation, and remodeling. Impaired healing exhibits defects in the regulation of one or more of these critical processes. We are currently investigating the regulation of inflammation in acute wounds versus that in chronic wounds which exhibit a prolonged inflammatory phase. We have previously found that Vascular Endothelial Growth Factor participates in resolution of inflammation through stimulation of Tumor Necrosis Factor
Superfamily Member 14 (TNFSF14/LIGHT). To determine the role of LIGHT in resolution of inflammation during wound healing in vivo, we performed wounding experiments in LIGHT-deficient mice. We found that in these mice, the re-epithelialized tissue is noticeably less mature than in wild type mice, with a discontinuous basement membrane and impaired epithelial-dermal connectivity. Re-epithelialized LIGHT−/− wounds also exhibit excessive inflammation, consistent with a critical role for LIGHT in the resolution of inflammation in vivo. We have also found that some LIGHT−/− wounds progress to form chronic, non-healing, wounds. These chronic wounds show more severe defective epithelial-dermal connectivity and increased inflammation than the acute wounds, as demonstrated by both a discontinuous epithelial basement membrane, and by the tendency of these wounds to re-open following re-epithelialization. Prolonged treatment with multiple anti-inflammatory and anti-bacterial components promoted the healing of these chronic wounds, implicating both inflammation and bacterial contamination in their chronicity. When we examined the bacterial content of the LIGHT−/− chronic wounds, we found that they were heavily contaminated with Staphylococcus aureus and epidermidis, two gram-negative species frequently found in chronic human wounds. The data from both acute and chronic LIGHT−/− wounds suggest a predisposition of LIGHT−/− wounds to become highly inflamed and bacterially contaminated, thereby increasing the propensity for development of chronic wounds. Our findings indicate that LIGHT−/− mice can potentially become a chronic wound model.

2326/B703
Differential Metabolic Labeling and Detection of Cell Surface Glycoproteins in Cultured Human T Lymphocytes.
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T lymphocyte subtypes can be characterized by the differential expression of cell surface cluster of differentiation (CD) markers such as CD3, CD4, and CD8. In many cases however the use of these cell surface markers can be ambiguous and it would be desirable to develop additional methods for use in differentiating cellular phenotypes. Differential metabolic labeling of cell surface glycoprotein subclasses may offer a new approach. It is possible to metabolically label specific subsets of cell surface glycoproteins including sialic acid containing, O-linked, and fucose containing glycoproteins by feeding cells unnatural tetraacetylated azide/alkyne-tagged sugars. Detection of labeled glycoproteins is accomplished by reaction with a fluorescent alkyne/azide partner probe in a copper-catalyzed “click” reaction. Human T-lymphocyte cultures were incubated with tetraacetylated azide/alkyne sugars for 48 hours, and the cells were fixed and click-labeled with azide or alkyne fluorescent probes. The cells were then incubated with fluorescently tagged antibodies against various CD markers. Labeled cells were analyzed by flow cytometry and fluorescence microscopy. for protein analysis, a fraction of the metabolically labeled cells were click-labeled with TAMRA azide or alkyne dyes and the labeled proteins were analyzed by 1-D gel electrophoresis. Our results demonstrate that cultured human lymphocytes efficiently incorporate tetraacetylated azide/alkyne sugars into specific subsets of cellular glycoproteins as demonstrated by 1-D gel electrophoresis and fluorescence microscopy. In addition, the click-based detection method can be multiplexed with CD marker detection as demonstrated by flow cytometry analyses. The flow results show that the total lymphocyte population exhibits markedly different levels of glycoprotein subclasses, and multiplexed analysis of the glycoprotein subclasses with CD markers may be used to uncover new T lymphocyte subpopulations. Finally, the metabolic labeling method can be used to track changes in cell surface marker expression throughout the lymphocyte activation and expansion process.

2327/B704
The Phosphoprotein PEA-15 Regulates ERK Localization and Function in T Cells.
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PEA-15 is a Death Effector Domain-containing phosphoprotein that binds ERK and restricts it to the cytoplasm. PEA-15 also binds to FADD and thereby blocks apoptosis induced by Death Receptors. Abnormal expression of PEA-15 is associated with type II diabetes and some cancers, however its physiological function remains unclear. To determine the molecular function of PEA-15 in vivo, we used C57 Black6 mice in which the PEA-15 coding region was deleted. We thereby find that PEA-15 regulates T cell proliferation. PEA-15 null mice did not have altered thymic or splenic lymphocyte cellularity or differentiation. However, PEA-15 deficient T cells had increased CD3/CD28-induced nuclear translocation of ERK, and increased activation of IL-2 transcription and secretion in comparison to control wild-type littermates. Indeed activation of the T cell receptor in wild-type mice caused PEA-15 release of ERK. In contrast, overexpression of PEA-15 in Jurkat T cells blocked nuclear translocation of ERK and IL-2 transcription. Finally, PEA-15 null T cells showed increased IL-2 dependent proliferation upon stimulation. No differences in T cell susceptibility to apoptosis were found. Thus PEA-15 is a novel player in T cell homeostasis. As such this work may have far reaching implications in understanding how the immune response is controlled.

2328/B705
The Role of Phospholipid Scramblase 1 in G-CSF-induced Granulopoiesis.
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Mice deficient in phospholipid scramblase 1 (PLSCR1) display reduced blood neutrophil count in neonates. Methylcellulose cultures reveal decreased colony numbers and colony size in PLSCR1-/- hematopoietic progenitor cells when stimulated with granulocyte colony-stimulating factor (G-CSF). Emergency granulopoiesis in response to In Vivo G-CSF administration is also attenuated in PLSCR1-/- mice. These previous findings suggest a supportive role of PLSCR1 in granulocytic lineage development. Given that granulopoiesis is essential for establishing innate immunity in mammals, PLSCR1 may play an important role in producing neutrophils from the bone marrow as part of host defense against environmental stress, including bacterial infection. Nonetheless, the molecular mechanism by which PLSCR1 contributes to neutrophil development is now unknown. In this report, pMSCV-retroviral transduction is used to introduce PLSCR1 protein in both primary and ER-Hoxb8 conditionally immortalized PLSCR1-/- bone marrow hematopoietic progenitor cells. Our results suggest that both endogenous and overexpressed PLSCR1 promotes granulopoiesis by facilitating granulocytic cell proliferation under G-CSF stimulation. Cell cycle analysis by BrdU incorporation in the ER-Hoxb8 immortalized cells also reveals a higher S-phase population in the PLSCR1 expressing cells at day 3 of G-CSF-induced differentiation. Additionally, prolonged expression of CCAAT/enhancer binding protein alpha (C/EBPα), a critical transcription factor for granulopoiesis, is observed in PLSCR1 expressing cells during G-CSF-induced granulocytic differentiation. In conclusion, our results suggest that PLSCR1 contributes to G-CSF-induced granulopoiesis, and this effect is associated with the prolonged expression of C/EBPα in the proliferating granulocytic cells.

2329/B706
Ubiquitination Regulates MHC Class II Recycling and MHC Class II-Peptide Complex Expression in Dendritic Cells.
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Elucidating the mechanisms regulating the expression and turnover of MHC class II-peptide complexes (pMHC-II) on the surface of DCs and therefore their capacity to activate CD4 T cells is an important biological problem. We now show that ubiquitination of pMHC-II by the E3 ligase March-I prevents the return of internalized pMHC-II back to the plasma membrane of immature DCs and promotes degradation of these retained complexes in lysosomes. DC activation very
rapidly terminates pMHC-II ubiquitination, and in mature DCs pMHC-II complexes recycle efficiently and are not degraded. pMHC-II in immature DCs isolated from March-I-deficient mice are expressed at very high levels of the plasma membrane and in these immature DCs pMHC-II degradation is also inhibited. Thus, the cellular distribution and stability of surface pMHC-II in DCs is regulated by ubiquitin-dependent inhibition pMHC-II recycling.

2330/B707

The Role of NADPH Oxidase in MAP Kinase Cascades Activation at Phagocytosis.

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The macrophage ability to engulf pathogens into specialized endocytic vacuoles and to destruct them is crucial for a proper immune response. For a long time the main mechanism of intraphagosomal pathogen destruction was considered as a result of an oxidative damage of pathogen cells by reactive oxygen species (ROS) produced by NADPH oxidase (NOX). The latest data provided a strong evidence that the pathogen destruction is a result of electrogenic NOX activity that makes an intraphagosomal environment optimal for phagocytic proteases activation. at the same time NOX family proteins are expressed in most cell types where they regulate various signal transduction pathways. Particularly, H2O2 produced by NOX oxidize cysteins in a catalytic cleft of protein tyrosine phosphates (PTPs) allowing tyrosine kinases to phosphorylate downstream targets like MAPKs. However, a correlation between the NOX activation in phagocytes and MAPKs activity remains unclear as well as a real time profile of H2O2 generation by a single phagocytizing cell. The aim of this research was to explore a real-time H2O2 production and the main MAPK cascades activation in phagocytizing RAW264.7 murine macrophages. Confocal ratiometric time-lapse imaging of H2O2 production in RAW264.7 phagocytizing cells expressing HyPer, a genetically encoded probe for H2O2, revealed a transient wave of NOX activation starting immediately after serum opsonized zymosan uptake and lasting for 15-20 min. To understand correlation between the H2O2 production and the Erk1/2 and p38 MAPKs activities, we used immunofluorescent staining and western blot analysis with specific antibodies to phosphorylated Erk1/2 and p38 forms. We observed both Erk1/2 and p38 pathways activation in phagocytizing RAW 264.7 cells. Erk1/2 but not p38 pathway activation requires NOX driven H2O2 production. Using a panel of inhibitors we demonstrated reciprocal p38 and Erk1/2 cascades inhibition in macrophages. Taken together, we revealed that the NOX function in phagocytes is nearly to be similar to that one in non-phagocytizing cell types. H2O2 produced by NOX is extensively used for the modulation of the signal transduction pathways by phagocytes.

2331/B708

WSB-2, a Novel Molecule That Suppresses the IL-21 Signaling.

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Interleukin-21 (IL-21) is a pleiotropic cytokine that regulates T-cell, B-cell, NK-cell, and myeloid-cell functions. IL-21 binds with its cognate receptor complex, which consists of the IL-21 receptor (IL-21R) and the common gamma chain receptor subunit. We identified novel IL-21R-binding molecules, WSB-2 which contains WD-40 repeats and a SOCS-box domain. Co-expression experiments of WSB-2 and various deletion mutants of IL-21R using 293T cells showed that WSB-2 associated with the membrane-proximal intracytoplasmic region of IL-21R, including box1 and box2. Next we examined the functional roles of WSB-2 on the IL-21R expression and on the IL-21-induced signal transduction. As the WSB-2's expression increases, IL-21R expression level and the IL-21-induced activated STAT3 that is main signal transducer of IL-21 signaling were decreased. on the other hand, small interfering RNA for WSB-2 enhanced the expression level of IL-21R, also indicating that WSB-2 negatively controls the receptor expression. To confirm the results of the 293T cell experiments, we used an inducible WSB-2 expression system (Tet-on
system) in mouse pro-B cell line, Ba/F3 cells. Induced WSB-2 by doxycycline significantly attenuated the protein level and also the surface expression level of IL-21R. Furthermore, the induced WSB-2 slightly but significantly reduced the IL-21-induced STAT3 phosphorylation. This study provides the first evidence that WSB-2 is the regulator of IL-21R expression and IL-21-induced signal transduction.

2332/B709
RhoA-Dependent Transcriptional Regulation of Hepatocyte Growth Factor Production in Response to Apoptotic cells.
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Background: Clearance of apoptotic cells by macrophages induces HGF secretion, which has been shown to play an important role in diverse cellular processes as cell survival, proliferation, migration, and differentiation in various organs. Objectives: We investigated regulatory mechanism at transcriptional step for HGF production in macrophages upon exposure to apoptotic cells. Methods: The activation of signaling molecules involved in transcriptional regulation of HGF production was determined following the exposure of RAW 264.7 cells to apoptotic or viable Jurkat cells. Results: Interaction of RAW 264.7 macrophages with apoptotic Jurkat cells, but not viable cells, and results in the induction of HGF production and mRNA expression. Exposure to apoptotic cells leads to activation of RhoA. Down-regulation of RhoA or RhoA/Rho kinase pathway by the siRNA for RhoA or pharmacological inhibitors blocked HGF mRNA expression as well as Akt, and MAP kinases, including ERK, JNK, and p38 MAP kinases. Inhibition of each of these MAP kinases also reduced HGF mRNA expression, but not Akt phosphorylation. Furthermore, inhibition of PI3-kinase decreased HGF mRNA expression and the MAP kinase phosphorylation. A blocking antibody against HGF receptor did not change apoptotic cell-induced increases in activation of RhoA, Akt and the MAP kinases as well as HGF mRNA expression. Conclusions: Our data provide evidence that direct activation of RhoA in response to apoptotic cells regulates mRNA expression and production of HGF through PI3-kinase/Akt/the MAP kinase signaling pathway.

2333/B710
XBP-1 and Blimp-1 Are Not Essential for the Conversion of Human Peripheral Blood Memory B Lymphocytes into Plasma Cells Induced by 2Methoxyestradiol.
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2-Methoxyestradiol (2ME), an end-metabolite of 17beta-estradiol, is an antiproliferative agent that is currently being tested in clinical trials for B lymphocytes cancer treatment. We here analyzed the effects of 2ME on normal human peripheral blood B lymphocytes. We report that sub-cytotoxic concentrations of 2ME induces the conversion of CD138- B lymphocytes into CD138+ cells of phenotype similar to immunoglobulin (Ig)-secreting plasma cells. Normal human B lymphocytes expressing CD138 increased in response to 2ME in a dose-dependant fashion, from 2% at baseline up to 31% in cells cultured in the presence of 0.75 µM 2ME. Moreover, most of the converted cells were also CD27+ and secreted high levels of IgG (151 µg/106 cells/24 hours). We then exploited this effect of 2ME to gain further insights into the molecular mechanisms that govern changes in transcription factors involved in plasma cells differentiation. Plasma cells generated by 2ME treatment of normal human B lymphocytes expressed elevated levels of IRF4 and reduced levels of Pax5. However, levels of XBP-1 were reduced, while no changes in Blimp-1 expression could be observed. Our results suggest that the differentiation of peripheral blood B lymphocytes into plasma cells does not require increased expression of XBP-1 and Blimp-1, in contrast to what has been shown for tonsil and bone marrow B lymphocytes. We hypothesize that 2ME-treated human peripheral blood B lymphocytes differentiate into short-lived plasma cells, and that this conversion is independent of XBP-1 and Blimp-1.
Macrophages can play pro and anti-inflammatory functions during muscle injury and recovery. In the present study, In Vivo and In Vitro models were used to determine the role of macrophages in muscle atrophy and regrowth. In the In Vivo model, contractile properties of soleus muscles were measured in mice depleted in macrophages (etoposide, 15 mg/kg) and submitted to a hindlimb unloading and reloading protocol. Ambulatory mice were used as control. In the In Vitro model, large C2C12 myotubes were incubated for 2 days in low serum medium (DMEM 2% horse serum) and co-cultured with macrophages that contained apoptotic neutrophils (anti-inflammatory macrophages) and/or insulin growth factor-1 antibody (anti-IGF-1, 5 μL/mL). Myotube diameter was measured in light microscopy. Protein content was quantified with BCA assay and myosin heavy chain content was determined by western blot. In Vivo experiments showed that mice depleted in macrophages had roughly a 30% decrease in maximal muscle force compared to matched placebo mice at 7 and 14 days post-reloading. Alternatively, In Vitro experiments showed that the presence of anti-inflammatory macrophages completely prevented the loss of protein content as well as the myotube atrophy after 2 days in low serum medium. Furthermore, the myosin heavy chain content of anti-inflammatory macrophages and myotubes co-culture had increased by 10% after 1 day in low serum medium, but had decreased by 10% in the myotube monoculture. Interestingly, the addition of anti-IGF-1 to the co-culture significantly decreased the ability of anti-inflammatory macrophages to protect against the myotube atrophy and the decrease in myosin heavy chain content after 2 days in low serum medium. These results clearly indicated that macrophages with anti-inflammatory phenotype prevent muscle atrophy while promoting muscle recovery. The protective effect of this type of macrophages is partly mediated by the release of IGF-1. Supported by grants from NSREC and CIHR.

Pre-College and College Science Education (2335 – 2357)

Use of Case Studies in Undergraduate Supplemental Cell Biology Instruction Sessions.

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According to the National Research Council’s meta-analysis completed in 2005, there are three main principles to the ways students learn science and should be used to drive science education reform, they are as follows: “1) coming to the classroom with alternative conceptions of ideas which are based on their life experiences and are resistant to changing these views, 2) developing competency of a discipline requires a deep foundation of factual knowledge that is organized in a conceptual framework, 3) students can learn to take control of their own learning through metacognitive approaches that define their learning foals and help them assess their progress (NRC 2005).” The importance of active learning has been established and many students are receptive to the idea of learning through case studies. The central dogma of science education, if you will, can be thought of as active learning→problem-solving skills→critical thinking (Paul and Elder 2001). The problem lies, as Gupta (2005) demonstrates, that few “basic-studies” or introductory science courses provide students with adequate opportunities to engage in active learning, develop problem-solving and critical-thinking skills, in addition to requiring logical thinking on behalf of the student.

Single Nucleotide Polymorphism (SNP) Mapping of Mutants in Undergraduate Molecular Biology Course.

- 1126 -

2009 ASCB Regular Abstracts
Science education is moving towards more authentic and inquiry-based approaches to enhance learning. Laboratory courses that provide genuine research experiences are one strategy for generating excitement and enthusiasm. This approach has been used in a one-quarter upper-level majors Molecular Biotechniques course by performing a C. elegans mapping project. First third of the class, the students are introduced to and perform the techniques necessary to map the molecular lesion of a mutant to a chromosome. These techniques include isolation of genomic DNA, PCR, and restriction digestion of restriction fragment length polymorphisms (RFLPs) also called Snip-SNPs. In addition, the students learn about the broader significance of single nucleotide polymorphisms (SNPs) in phenotypic variation of organisms and disease by identifying examples through literature searches of primary research papers. In the last three weeks of the course, the students apply theses techniques to map a mutant previously isolated in a genetic screen. Each student was given an F2 recombinant from a cross between the mutant (in the N2 background) and the polymorphic mapping strain CB4856, with the goal of collectively mapping the mutation to a chromosome. The students isolated genomic DNA, cloned Snip-SNP fragments for each of the six chromosomes, and digested the Snip-SNPs using a single restriction enzyme, Dral, common to each of the PCR fragments (Davis et al. 2005 BMC Genomics 2005, 6:118). on the last day of the class, the students compiled their results together as a class. The class data was analyzed and suggested that the mutation is on Chromosome V. Students’ comments indicate that they enjoyed the challenge of such a project, especially when their experiments lead to novel research findings. The novelty of the project can be maintained by using a new mutant each year.

2337/B714
Interdisciplinary Teaching and Learning in Biophotonics.
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Light provides a means to image, analyze, and manipulate living cells and tissues. Photonic applications have produced a wave of new discoveries that have revolutionized our understanding of a vast array of biological processes. The exceptional scientific breadth of biophotonics research makes it virtually impossible for a single science department to deliver all topics necessary for a well-rounded basic undergraduate education in this rapidly progressing scientific area; therefore, we developed a new interdisciplinary course. The instructors are from the Departments of Physics, Chemistry and Biological Sciences and the students come for each of those departments as well. The course includes lecture, discussions, team activities and projects, and practical experiments with organic fluorophore synthesis, advanced spectroscopy, and the use of microscopy in cell biology. Students work in interdisciplinary groups, which fosters peer-to-peer learning and develops their communication skills in a cross-disciplinary setting. Students produce a portfolio which is used for assessment and they present final team projects dealing with the synthesis of organic dyes and their properties In Vitro and when introduced into living cells or projects dealing with the three-dimensional visualization of fluorescently labeled cells. Pre- and post-assessment of student viewpoints revealed changes in attitude and perceptions. for example, as expected by the opportunities given in the course, more students indicated that they had “designed, or worked with others to design, an experiment to test a hypothesis”. Following the course, there was an increase in the number of students who felt that they “enjoy and feel comfortable working with others in lab settings to solve problems together”. Student attitudes toward team teaching by several instructors became more positive after the course, with a greater number of students feeling comfortable with a team-teaching approach. Students agreed more strongly after the course that they saw “relationships among topics discussed in chemistry, biology and physics”.
An Advanced Cell Biology Course Designed for Undergraduates to Propose, Conduct, and Publish Original Biomedical Research.
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Undergraduate educators face significant challenges in preparing diverse graduates for a scientifically sophisticated and interdisciplinary 21st century community. Science curricula that integrate training in research with undergraduate teaching enjoy much funding support. The goal of providing comprehensive original research experiences within a structured course is a particular challenge, but was achieved in the course described here. Students conducted individualized projects integrated to budding yeast and fission yeast models developed by my research laboratory for studying the toxicity of Parkinson’s disease protein alpha-synuclein (Sharma et al., J. Mol. Neurosci. 28, 161-178, 2006; Brandis et al., J. Mol. Neurosci. 28, 179-192, 2006). The potential to discover new modes of molecular regulation of alpha-synuclein toxicity provided the impetus for student-driven discovery. First, each student picked a “my favorite yeast gene” (MFG) or “my favorite alpha-synuclein mutant” (MFM) to study. Students then organized and led in-depth discussions to familiarize each other with MFG/MFM background. Next, they uncovered gaps in knowledge by presenting journal clubs that bridged their MFG/MFM with PD pathogenesis. Next, students wrote grant proposals to conduct original research based on the technologies available to them at the college, and spent the first six weeks mastering these techniques. They spent the rest of the semester conducting experiments, interpreting data, and communicating their work. Instead of a final exam, students presented their research at a “Frontiers in Parkinson’s Disease” conference and published papers in an online undergraduate peer-reviewed journal (see EUKARYON poster at this meeting). Student research also yielded at least one of these outcomes: 1) publication in a national undergraduate research journal; 2) presentation at an external scientific conference; 3) preliminary data for a successful NIH grant. Such courses, while necessarily small and self-selective, provide successful authentic research experiences that prepare undergraduates for professional scientific careers. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

Fishy Friends, Science Research and a Boost for Education.
M. Fields, T. Neuroscience Research Course; Sidwell Friends School, District of Columbia, DC

Neuroscience Research has been an elective course at Sidwell Friends School since the mid 1990’s. Research projects of students in the course include thigmomorphogenesis in plants, communication in insects, neurophysiology and neuroethology of weakly electric fish, and the genetics and development of zebrafish. One focus of our work is the establishment of collaborative relationships with scientists whose research focuses in our areas of interest. This effort has included collaboration with the National Center for Microscopy and Imaging Research at UCSD and, most recently, the Stephen Ekker Lab at Mayo Clinic in Rochester, MN. Another focus of our work has been the development of teaching modules to be shared with other schools. This year, a small subset of students who have been involved with research projects throughout their careers in the Upper School are designing and teaching their own modules in this course. There are nine student developed modules including lab protocols on action potentials in worms and crayfish; fin regeneration in zebrafish; the effects of ethanol on the development of zebrafish; chromatophores and pigmentation in zebrafish; and avoidance behavior and learning in zebrafish. Several student modules have been published in a special issue of the Zebrafish Journal. This course concludes with the presentation of our research at the ASCB annual meeting.

Not Just in Time: Using a Preparation/Participation Scaffold to Transform Student Seminars.
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Literature-based seminars introduce students to the community of science and the scientific process in the most direct way short of actually working in the field or laboratory on independent research projects. Two common challenges in seminar courses, however, are that students don’t know how to read an article in the primary literature critically or how to make substantive contributions to the discussion of the articles that they read. The learning impact of the seminar is reduced in proportion to the number of students who come to the seminar underprepared and/or don’t participate actively in the discussion. We have developed a general, iterative assessment strategy that addresses both of these issues and has made a dramatic impact on the quality of the seminar courses we have taught. A scaffold consisting of a set of conceptually related articles to critique, seven common questions to explore when reading and critiquing a scientific article, and a regular cycle of two-way feedback related to student work in the course, promotes student preparation and participation, both of which have approached 100% in this style of seminar. Student ratings of these seminar courses have also been exceptionally high suggesting that students appreciate the effectiveness of this format of seminar and are willing to accept the additional work that the format requires of them.

2341/B718
Incorporating Fluorescence Microscopy into Both the Cell Biology Laboratory Class Experience and Undergraduate Research with Students in a Science Teacher Preparation Program.

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Fluorescence microscopy has become a critical tool for most cell biologists. In the past, many biology majors solely learned about cell structure through the use of prepared slides of fixed cells stained with various dyes. While these techniques are still valuable, we created a fluorescence microscopy module to help cell biology students learn about newer methods for studying cell structure and protein localization. We also presented fluorescence microscopy as a means to not only study cell structure, but to follow changes in cell structure in a living cell over time. Cell biology students learned about the major features of the microscope essential for performing fluorescence microscopy, the use of green fluorescent protein (GFP) and its variants in cell biology research, and observed samples expressing fluorescent probes highlighting major parts of the cell. Students completed questionnaires after the module, and overall found that the module increased their understanding of fluorescence microscopy and its use in cell biology research. In addition to the use of fluorescence microscopy in the cell biology laboratory class, we worked with an undergraduate science educator-in-training to develop a fluorescence microscopy teaching tool geared towards high school students. This project involved using budding yeast strains expressing several kinds of fluorescence probes specific to different organelles. The undergraduate researcher captured images and time-lapse movies using the various budding yeast strains, and integrated them into a lesson on cell structure and organelles following the state curriculum guidelines. We presented this project to several groups of high school students in New Jersey. Students completed questionnaires about the presentation, and the results indicated that the high school students increased their understanding of cellular organelles and the use of fluorescence microscopy in research.

2342/B719
Crayfish Electrophysiology Experiments for High School Students.

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High school students are rarely exposed to electrophysiology experiments. This experiment uses crayfish as a model organism for studying electrophysiology. Crayfish can be inexpensively obtained from biological supply companies and have a well-studied nervous system. In this experiment, action potentials are recorded from the nerve cord of crayfish. The effects on
spontaneous nervous activity of changes in temperature, electrical stimulation, and pharmacology using various neuroactive drugs can also be observed. To record from the nerve cord of crayfish, the organism's abdomen is first removed from the rest of its body. The exoskeleton is subsequently removed to reveal the nerve cord. The preparation is bathed in saline, and a suction electrode is used to record action potentials. The suction electrode is connected to a biological pre-amplifier, which is then connected to an oscilloscope. This electrophysiology experiment is a useful avenue for exploring neuroscience at the high school level, including topics such as action potentials and the pharmacology behind neuroactive drugs.

2343/B720
The HHMI Science Education Alliance’s National Genomics Research Initiative: A Model for Undergraduate Science Education.
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A goal of Howard Hughes Medical Institute’s Science Education Alliance (SEA) is to have research scientists and science educators collaborate to improve the production and quality of 21st-century scientists. The SEA’s first offering is the National Genomics Research Initiative (NGRI), a collaborative in which a scientist poses a question of the year and networked faculty across the country direct college freshmen in an authentic research experience as part of their curriculum. An objective is to expose novices to the process of doing science. The NGRI course is based upon Graham Hatfull’s Phage Hunters program. In its inaugural year, 12 diverse institutions implemented the course either as a substitute for or in addition to the standard introductory biology laboratory course; targeting their typical entering freshmen, freshmen classified as at-risk, or freshmen admitted into the honors college. Results- These 270 students used techniques in microbiology, molecular biology, and electron microscopy to isolate and preliminarily characterize 234 new mycobacteriophage and a unique bioinformatics workflow to annotate the 12 sequenced phage genomes. By the end of the academic year, eight completely annotated genomes were submitted to GenBank. In this first year, we observed: 1) higher student retention in the NGRI course relative to the standard introductory biology laboratory course (94% vs. 88%); 2) higher introductory biology lecture course average (6 points) than non-NGRI students; and 3) gains in student behavior and attitudes about science consistent with national trends. Conclusions- The data suggest that our model (a) addresses the suggested standards for demonstrating science proficiency outlined for pre-college education (Duschl RA and Schweingruber HA, eds. “Taking Science to School: Learning and Teaching Science in Grades K-8”, 2007, http://www.nap.edu/catalog/11625.html) that is applicable to all education levels and (b) produces positive results regardless of the target freshman population, implementation strategy, or resources available at the institution.

2344/B721
Fostering Interdisciplinary Teamwork in an Undergraduate Biological Databases Course.
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To prepare students for future careers in 21st century biology, we have explicitly taught them how to carry out interdisciplinary research within the context of an undergraduate course on Biological Databases, team-taught by a biologist and a computer scientist and cross-enrolled by students from both majors. The learning goals were: 1) to understand how biological information is encoded in the genome and to apply this knowledge to a variety of biological problems; 2) to understand the core concepts, structure, and functions of a database, ranging from individual files to a full relational database management system, and to perform useful tasks with such data; 3) to show discipline and proficiency in day-to-day science and engineering best practices, such as maintaining lab notebooks, managing files and code, and critically evaluating scientific and
technical information; 4) to build skills and tools for “leaving your comfort zone,” flourishing outside of it, and learning more about biology and computer science on your own, and 5) to learn how to effectively communicate and work with colleagues from different disciplines. Students submitted weekly homework assignments and reflections to a course wiki. After the final project, the students were grouped in teams of four to create GenMAPP-compatible Gene Databases for four new species that were not yet available, using XMLPipeDB, an open source tool chain for building relational databases from XML sources. Students in each team were assigned the roles of project manager, coder, ID minder, or GenMAPP user. The project manager was responsible for managing the team wiki and setting project milestones for the deliverables. The coder made any needed modifications to the XMLPipeDB code. The ID minder tested the exported Gene Database for data integrity, comparing it with the UniProt and Gene Ontology source data. The GenMAPP user then analyzed a publicly available DNA microarray dataset using the team’s new Gene Database. Each team also created documentation for their deliverables and wrote a group paper. Three students continued the research project beyond the course, leading to the public release of Gene Databases for *Vibrio cholerae* and *Plasmodium falciparum*.

**2345/B722**

**Research to Teach the Masses: Large Scale Implementation of Research Modules in the Undergraduate Teaching Laboratory.**  
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According to NSF’s Science and Engineering Indicators underrepresented students earn only 6% of all science or engineering Ph.D. degrees awarded in the US. Today, more minority students are entering undergraduate programs than ever before. Despite this fact, this population remains underrepresented at every stage in the academic pipeline. Many studies suggest that hands-on research activities enhance students’ interest in pursuing a research career. The prevailing question is how to engage the research community to encourage students to pursue a research career. In primarily teaching institutions where access to research is limited, this challenge is even greater. Here, we present a model for the large-scale implementation of laboratory research in the undergraduate teaching laboratory. Cassava, an economically important crop, is a staple food in Africa, Asia, South and Central America. Despite wide consumption in the Caribbean region, the genetic diversity is poorly understood and basic cellular characterization as it relates to starch and amylase content has not been performed. Lab modules, broadly entitled ‘Bring Your Own Cassava’ (BYOC), were implemented in upper division genetics and cell biology courses. Students enrolled in these courses are contributing to the understanding of cassava lineage and nutrient composition. Samples are brought by students from their respective towns and compared against known lineages from local agricultural stations. Assessment of content and learning perceptions revealed that our novel approach allows our students to learn while engaged at multiple levels in our research endeavor in characterizing cassava in Puerto Rico. Our proposed method could enhance student’s competitiveness by providing them with valuable research skills. In the short term, these skills could benefit the PI in the undergraduate research laboratory, while in the long term could benefit a prospective graduate mentor.

**2346/B723**

**Stepping Away from the Podium: Transforming Biology Majors’ Introduction to the Foundations of Biology by Engaging Them as Colleagues.**  
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An optimal college biology program offers its majors a transformative first experience with biology that incorporates best practices for learning and provides the foundation for students’ professional success. Undergraduates in the College of Biological Sciences at the University of Minnesota first
engage the fundamentals of the discipline in a year-long introductory course with lab, called Foundations of Biology. Instead of attending lectures, however, the 117 students in each section meet twice a week for two-hour activity- and project-focused “Concept Labs” (D. Udovich, Oregon). These sessions occur in a technology-enabled, SCALE-UP-style classroom (R. Beichner, NC State) that facilitates student collaboration during class. Key features of the room include round tables for groups of nine and table-dedicated LCD monitors, white boards and laptop computers. All course activities emphasize collaboration and higher order cognitive skills (Bloom’s levels: application, analysis, synthesis, and evaluation/creation). Accompanying labs give students the tools and experiences to approach novel research questions and culminate with students designing and carrying out an independent research project. The courses emphasize: formative assessment; immediate feedback; group discussion to arrive at understanding; opportunities to revise work based on feedback; explicit integration of University and College learning outcomes; and regular use of critical reflections and concept maps to make the emerging accuracy and complexity of student understanding apparent. Students learn to be accountable for their preparation, identify reliable sources, design original research projects, develop quantitative skills, apply their knowledge to case studies, interpret data, hone their writing and oral presentation skills, and assess their peers’ work. within this model, course instructors serve as coach and colleague rather than primary information provider. Preliminary analysis of early cohorts that completed these courses suggests they outperform other students in lecture-based upper division courses. We will report our analysis of the impact of Foundations of Biology on student attitudes, performance, and retention in science.

2347/B724

An Introductory, Interdisciplinary Chemistry-Cell Biology Course Sequence at St. Olaf College.

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Recognizing that science has become increasingly interdisciplinary, the Chemistry and Biology Departments at St. Olaf College sought to develop an introductory sequence of courses that could replace three departmental courses: two semesters of general chemistry and a semester of introductory biology (cell biology and genetics) that are currently taken by all biology majors and the majority of chemistry majors. Three new interdisciplinary courses were developed. The three new courses, like the departmental courses they replace, include laboratory. Unlike the courses they replace, the three new courses integrate material from both chemistry and biology. The Fall term course, Fundamental Chemical Concepts and Their Biological Applications, includes topics in atomic structure, bonding interactions, water and its solutions, biological membranes, protein structure, chemical reaction types, stoichiometry, equilibrium systems, and acids and bases. The January term course, Chemical Thermodynamics, Kinetics and Biological Relevance, includes topics in probability as the driving force for reactions, the relationship between bonding energy and equilibria, oxidation-reduction reactions and electrochemistry, and rates of reactions, including enzyme-catalyzed reactions. The Spring term course, Molecular and Cellular Biology, includes topics in cell structure, metabolism, movement, signaling, division, and molecular and Mendelian inheritance. Objectives for the three new courses include: presenting biological and chemical material in an integrated way; covering essentially the same content as the departmental courses; increasing student interdisciplinary thinking; and providing a route by which our students can complete introductory material in both biology and chemistry in their first year of college, thus allowing them easier access to a wider variety of upper level courses. The new courses have now been taught for two academic year cycles, and we will present evidence that these goals are being met. We will also present information about course content and the laboratory components as well as lessons learned and modifications that we have made to the courses based on our assessment.

2348/B725

K-12 Science: Partnerships for Change.

Student performance in US science is dramatically below key expectations for long-term success of the American economy. For example, only 38% of eighth graders scored “proficient” on the 2008 Minnesota Comprehensive Assessment (MCA) in science. Science curriculum must be re-imagined and aligned with new education standards to address this major ‘science gap’. We established a new program to rebuild science K-12 education curricula for the 21st century. We began with a tripartite partnership between the Mayo Clinic (life science expertise), Winona State University (teacher education expertise), and the Lincoln K-8 Choice Rochester Public School. An initial group of 18 Lincoln teachers plus a zebrafish science educator from Sidwell Friends School (Washington, D.C.) worked in the lab in the summer of 2009, conducting authentic genetics and developmental scientific research using the zebrafish (Danio rerio) model organism. The teachers then worked as teams to incorporate this cutting-edge research into new curriculum modules for the upcoming year. Winona State University provided graduate credit in Education and modules and materials from a local Science Lending Room. Eighth-grade students participated in the final week of the externships, returning critical student insights on how to improve these new teaching modules. The success of this work can be attributed to a full exchange between authentic science practiced by leading researchers that enables great teachers to build state-of-the-art science curriculum. Future goals include expanded partnerships in engineering for cutting-edge physical science K-12 curricula and to expand the use of these new teaching modules elsewhere to address the US science gap.

2349/B726
Peer Instruction or Gaming the System: Does Displaying Class Results Affect Quality of Student Discussion?
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We are studying strategies for implementing peer-learning in the Introductory Biology classroom. at UW-La Crosse, this class consists of 8 sections of approximately 100 students. One common peer-learning strategy is the think-pair-share (TPS). We often use student response units (“clickers”) to assess student understanding of a concept. Our clicker system allows us to display student responses in graphical format. This experiment was designed to determine if there is a difference in the quality of peer discussion and student behavior depending on whether they have seen the graph of student responses. We hypothesized that students would learn through peer-discussion and not simply move to the most common answer shown after initial class responses. Methods: We asked 18 questions half of which were true/false and the other half were multiple choice. In one treatment students were shown the class responses while allowing peer discussion, in the other treatment students did not see the class responses during discussion. Each group of students had nine of the questions in treatment one and nine questions in treatment two. Results: In both treatments we saw a significant number of students changing their answers to the correct answer. When students saw the graph of initial responses, they were 30% more likely to change their answer to the most common response (than the students who just discussed the question with their neighbours). Additionally, we observed that students who finished the course with a grade of C or D were more likely to switch to the most common answer if shown the initial response graph. Conclusion: Our data points to students “gaming” the system by choosing the most common answer shown during discussion time. We recommend against showing class responses prior or during peer discussion.

2350/B727
Research Experiences for Undergraduates: Future Teachers Develop Scientific and Mathematical Thinking Skills & Strategies for Teaching K-12 Students.
Research experiences in scientific laboratories are crucial for reaching a deep understanding of the nature of the scientific enterprise. Without actually experiencing how research is done, it is difficult to understand how to formulate testable questions, collect and analyze data, create models to explain results, or to communicate conclusions that are strongly supported by evidence. In particular, science and math teachers must experience research themselves in order to guide their students to develop skills in scientific thinking and mathematic analysis, and to effectively solve problems and perform experiments. Building on previous work that defined instructional and assessment tools for encouraging high school science students to develop specific skills for engaging in scientific research, we designed a summer research program for undergraduate science and math majors who are committed to teaching in urban K-12 schools. As part of the Cal Teach program, they participate in Department of Energy-sponsored Pre-Service Teachers (PST) research internships under scientist mentors at the Lawrence Berkeley National Laboratory. During the 9-week program, Cal Teach students engage in full-time research in their field, and participate in a course that aims to help them connect the advanced concepts that they are learning in their laboratories with their future teaching in the K-12 classroom. Students are supported to create research proposals and scientific posters, and develop K-12 lesson plans that align with their research. Each week the course explores a theme that represents one step in the research process, and strategies for integrating science and math concepts are made explicit. Student work was analyzed and pre/post tests were administered to measure how student understanding of scientific research changed from the beginning to end of the program. Using a Knowledge Integration framework to score responses, we present evidence that students further developed their own scientific and mathematic thinking skills, learned how to guide their future K-12 students to engage in scientific and mathematic thinking, and applied scientific and mathematic inquiry to their own teaching practices.

2351/B728

Methods to Assess Retention and Research Programs for Underrepresented Science Students: An Analysis of PEERS at UCLA.

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The Program for Excellence in Education and Research in the Sciences (PEERS) is a two-year program for freshman/sophomore students at UCLA. All PEERS students are underrepresented life/physical science majors that have had more than typical life challenges to overcome to reach college. PEERS' goal is to retain these students in science, bring them into the scientific community and encourage them to try undergraduate science research. The PEERS program involves individualized academic and career counseling, "Pathways in Science" seminars tailored to freshman and sophomore needs, supplementary instruction through Treisman-style collaborative learning workshops, research seminars by UCLA professors, and the opportunity to both be exposed to and to be deeply involved in undergraduate research. We have used both survey and post-matching assessment methods to evaluate the efficacy of PEERS' programmatic elements. Assessment surveys have confirmed that the "Pathways in Science" seminars have assisted the students in reaching their academic and career goals. Assessments using post-matching controls have revealed that PEERS involvement increases both the likelihood of entry intro research (61% PEERS vs. 19% control) and the length of time students spent in research (median 8 quarters PEERS vs. 2 quarters control). Further we find that the identification of appropriate control groups is necessary to discern the true efficacy of programmatic elements, as the use of an inappropriate control group can lead to biases in interpretation. An analysis of the efficacy of the Treisman-style workshops revealed that using a less-constrained control group made it appear that all the workshops were successful with the PEERS students receiving the expected 0.5 unit grade increase. Using a more constrained control group, with features more closely matched to the PEERS students with regards to preparation for college (as gauged by
Math SAT score and other factors), revealed that workshops accompanying Math courses were successful in improving PEERS student’s grades but workshops accompanying Chemistry courses were not. In summary, it is critical to use an appropriate assessment method when evaluating undergraduate science curricula.

2352/B729

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Researchers and educators around the country are creating courses and materials that teach students at the undergraduate and early graduate level how to describe and model cellular systems in quantitative and predictive ways. CompCellBio was built as a project development site for researchers and educators to collaboratively create teaching modules that could be integrated into existing undergraduate biology courses. The CompCellBio Web site is part of the Twiki™ hosted by the R. D. Berlin Center for Cell Analysis and Modeling. A Twiki allows individuals to view, edit, store, and manage changing documents via web browsers which makes authoring web content independent of location or computer platform. As a site for developing teaching materials, the goals of the site are to make it easy for users to contribute, find and retrieve materials. A template teaching module based on an outline proposed by researchers who use mathematical models to study cellular systems is provided. The outline includes: descriptions of physiology, molecular biology, the mathematical model, as well as model files, exercises, and references. Five teaching modules are currently available including such topics as the cell cycle, circadian rhythms, and reaction kinetics. As modules are added new features (tables or categories) are identified that could improve the CompCellBio Web. For example, the course "Modeling in Biology" contributed by Mogilner and Dey required the creation of additional categories. Current modules make use of one of three software applications: XPP/WinPP, Virtual Cell, or Matlab. Faculty are invited to make use of the existing content, develop their own topic modules, and make or request changes that make the content and features of CompCellBio web better address their needs.

2353/B730
A Cell Biology Education Online Group Project.

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This educational project takes advantage of the recent growth in online journal access, educational resources and online interactive editing. A University of New South Wales undergraduate science course in cell biology (ANAT3231) has three main forms of assessment: individual, group and theory. For several years the group project had been a group prepared and presented poster on a group selected cell biology topic. The new group project was the preparation of an equivalent online cell biology topic by 10 groups of 4 randomly selected students, as would occur in any future research environment. A Mediawiki server was installed locally, a new site established (http://php.med.unsw.edu.au/cellbiology), and access controlled using the existing university LDAP system. Students selected a topic from a range of titles (http://php.med.unsw.edu.au/cellbiology/index.php?title=ANAT3231_Projects_2009), time was allocated in each week's practical class for group project work and projects could be worked on at any time outside class hours. Part of this laboratory time was allocated to instructor tutorials on: assessment criteria, reference searching, copyright, image uploading and online editing. Individual projects were designed as components of the group project. All online content is accessible, viewable and printable by all. Only currently enrolled students can edit content and also remain generally anonymous by student ID number. All project edits are automatically logged, can be undone and individual contributions can be observed and assessed. A separate discussion page was available for group planning and interactions. After submission date, peer assessment and online comments were made of each project and the group was able to improve
Science proficiency nationally is a major challenge in K-12 education. For example, only 46% of Minnesota eighth graders scored "proficient" on the 2009 Minnesota Comprehensive Assessment (MCA) in science. The science education approach including the current curriculum and teacher proficiency must be re-imagined and aligned with new education standards to address this major 'science gap'. We established a new program, InSciEd Out: Integrated Science Education Outreach, to rebuild K-12 science education curricula for the 21st century. The program is based on partnerships between schools and local industries to update the science of teachers and bring available technology and mentorship to the classroom. The program is discussed in another abstract. Here we introduce the product: horizontally integrated, vertically aligned teaching modules. Our teaching modules consist of classroom activities organized into Roger Bybee’s 5 E’s of constructivist instruction. The 5 E’s: Engage, Explore, Explain, Extend, and Evaluate in InSciEd Out are represented by lessons and activities that would traditionally be found segregated to individual subject course work. Our modules include mathematics, reading, discussion, history, and social studies focused on a particular topic thread inspired by a set of current Education Standards. Science experiments may be found at any level within the 5 E’s. All modules include mentorship from scientists currently involved in the field of study being taught. These modules, once vetted in pilot schools (occurring in school years 2009-2011), will be made freely available through our website http://sites.google.com/site/insciedout/. Our progress will be shared on this same site with discussion forums for continued improvement. Authentic science education requires that learners experience science as they find it in their world: fully integrated with all other subjects. InSciEd Out represents a new partnership paradigm where curriculum change is empowered by intellectual and technical resources through partnerships of strengths to address this major educational challenge.
curriculum and its community of student scholars. We found that Eukaryon contributes most significantly to the preparedness for future careers of its board members and provides them with a better understanding of the scientific publication process. Our current efforts focus on further strengthening the familiarity of Eukaryon with non-major and major students and improving communication with the faculty on our submission and review process. Finally, as another measure to assess impact, we report an analysis of our online readership using data from Google Analytics. Given the rapid success Eukaryon has enjoyed at our institution, we encourage the adaptation and implementation of such journals at undergraduate institutions that seek to further strengthen their community of students as scholars and their inquiry-based pedagogy. (Supported by NSF-CCLI)

2356/B733

**Immune Attack, a Video Game in the Molecular World.**

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Jerome Bruner proposed the Spiral Curriculum as a method for creating deep understanding of scientific concepts. Bruner writes that five year olds intuitively create grammatically correct sentences and are therefore prepared to understand sentence structure more deeply when it is formally introduced in school. Immune Attack is a three-dimensional video game that provides a place to gain an intuitive understanding of cellular biology and molecular science. Proteins, molecules and cells behave in Immune Attack as they do in nature. Objects in the game, such as white blood cells, are drawn to look like the schematics that scientists use in their own models. Game actions, such as the capture of white blood cells by proteins on blood vessel walls, mimic actions that occur in nature, and are described using vocabulary similar to that of scientific literature. Our Scientific Advisory Group of 20 active scientists reviews our game outline and contributes to the faithful and exciting presentation of molecular science. We have successfully developed a method of communication, a guided wiki-like document, that allows these scientists to contribute meaningful information in a time efficient manner. We collaborate directly with teachers to conduct controlled evaluations, using the Medical Mysteries Series (MedMyst, which covers non-molecular aspects of infectious disease) as a negative control. We tested students' knowledge, comprehension of game dynamics and confidence with the material (n = 180 Immune Attack, n = 142 MedMyst). We found highly significant gains in confidence with molecular science related materials and significant gains in knowledge of the cell biology and molecular science presented, in the groups that played Immune Attack as compared to the control game. We are building on the results of these studies to create an evaluation tool for the Fall of 2009. Additionally, we have used Immune Attack to inspire high school computer programming classes to create their own new videos games based on Immune Attack. Immune Attack provides an intuitive introduction to molecular and cellular biology for 7th grade students, thus significantly lengthening the “Spiral Curriculum.”

2357/B734

**Developing Scientific Thinking in a Virtual Wet-bench Environment.**

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We have created a virtual wet-bench environment that enables exploration of basic biological principles at the tissue, cell, and sub-cellular levels through experimenting with computer models. By conducting experiments and analyzing data, students develop problem solving skills, and an empirical understanding of how evidence, logic, skepticism, explanation, and models are used to formulate testable hypotheses in cell biology. Learned skills and topics of study overlap with content standards for secondary science curricula or constitute core material of college-level cell biology courses. These topics cover basic principles, including tissue development, homeostasis, cell signaling, gene expression, metabolism, growth, division, death, and natural selection, as well as more specialized processes, such as tumorigenesis and host-pathogen interactions. Simulations created with a research-oriented computer-modeling platform are accessible through

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a user-friendly graphical interface developed with feedback from high school and college-level science educators. A library of models supports novel experimentation, recapitulation of classic experiments in cell biology, or demonstration of standard experimental techniques. By incorporating abstraction, experiments can be tailored to a wide variety of purposes and course formats across secondary and post-secondary curricula. For instance, one model may focus on exploring detailed genetic interactions controlling cell proliferation, while another version uses abstract representations of pathways, allowing students to appreciate cell-level phenotypes without getting lost in details. As they perform experiments, students can collect data and record observations in a virtual notebook, analyze data, and generate charts, graphs, and images of virtual cells to communicate their findings. The web-based interface also enables educators to customize and direct student experiences, and it fosters collaboration among educators by supporting collegial discussion and sharing of models, lesson plans, associated documents, and references.
Minisymposium 25: Cell Cortex and Membrane Dynamics (2358 – 2363)

2358
The Role of the Actin Cytoskeleton in Mitotic Cell Rounding.
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Animal cells, whether in a culture dish or in a tissue, undergo a complex sequence of morphological changes as they pass through mitosis. This begins at the onset of mitosis with retraction of the cell margin and cell rounding, and is followed after the onset of anaphase by axially cell elongation and cytokinesis. Here we describe our efforts to explore the role of cytoskeletal regulators in mitotic cell rounding and the molecular mechanisms that couple mitotic actin re-organisation to the cell cycle clock using Drosophila and human cells in culture as model systems. We show that mitotic cell rounding begins prior to nuclear envelope breakdown and is independent of microtubules. Using RNAi screening, we have identified a number of core regulators of actin dynamics that are involved in this process. This includes ERM-family proteins, Ezrin, Radixin and Moesin, which once activated by phosphorylation function to cross-link actin filaments to the cytoplasmic tails of plasma membrane proteins. The sole Drosophila homologue of these proteins, Moesin, is activated at the onset of mitosis and when artificially activated in interphase cells is sufficient to drive the re-organisation of cortical actin filaments to induce both rounding and cortical stiffening, independently of Myosin II. Conversely, in the absence of Moesin, Drosophila cells remain soft and flat as they pass through mitosis. Strikingly, these soft cells also exhibit profound defects in spindle morphogenesis and chromosome alignment, which can be rescued by re-establishing cortical tension from the outside the cell. These data reveal the importance of cortical stiffening and cell rounding as a prelude to chromosome segregation in animal cells, and in doing so help to explain the universality of this process.

2359
Cortical Instabilities Stabilize the Cleavage Furrow During Cytokinesis.
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The formation and ingress of the cleavage furrow during cytokinesis relies on a controlled reorganization of the actin cortex. Starting from anaphase, the cortical network redistributes from the poles to the cell’s equator and gives rise to the cytokinetic ring, which separates the two daughters cells. Strikingly, cleavage does not only result from localized constriction of the ring, but also requires coordination of cortical activities at the poles of the cell. In order to probe the role of the polar cortex during cytokinesis, we have mechanically disrupted the cortex by laser ablation and local delivery of actin-depolymerizing drugs. We found that these perturbations lead to oscillations of the cleavage furrow, which moves back and forth over the cell surface, resulting in eventual cytokinesis failure. Similar oscillations could be induced by depletion of genes regulating cortex turnover and activity. In all cases, oscillations seemed to be triggered by an imbalance in cortical contractility between the two poles of the dividing cell. Strikingly, high-resolution analysis of control divisions showed that small oscillations about the cleavage plane can sometimes be observed even in normally dividing cells, suggesting that the cleavage furrow is inherently unstable. In oscillating cells, we observed that the reversal of the direction of oscillation direction coincided with disassembly of the cortex and growth of blebs in the contracting part of the cell. Stabilization of the cortex by Wheat Germ Agglutinin led to oscillations, suggesting that a dynamic cortex is required for cleavage furrow stability. We propose that cortical contractility at the poles of the dividing cell and therefore intracellular pressure in the two daughter cells are tightly regulated and that, under control conditions, any pressure imbalance is buffered by destabilization of the cortex and the formation of blebs. When the polar cortex is stabilized, cortical oscillations can occur, leading to cytokinesis failure.
2360

The Cell Shape Changes of Cytokinesis.
D. Robinson; Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD

Cytokinesis, the shape change in which a cell divides, is fundamentally a mechanical process. To characterize the mechanical parameters of cytokinesis, we initially focused on how myosin-II motors and actin-associated proteins interact to control these mechanics using a variety of methods. We have also discovered a mechanical feedback controller that responds to cellular deformations. We have found that all of the features of myosin-II mechanochemistry and bipolar thick filament assembly are precisely tuned for this control system, though many of these same properties are expendable for cytokinesis itself. Furthermore, by studying the cellular response to applied deformation, we have been able to examine the requirements of cellular contractility independent of spindle signals. Finally, we have identified a pathway that includes the tumor suppressor 14-3-3, microtubules, and a Rac small GTPase (RacE), which modulates cortical mechanics and cytokinesis dynamics. Interestingly, 14-3-3 proteins are implicated in several human cancers and here we specifically link 14-3-3 to cytokinesis and cortical mechanics, two cellular processes where defects are linked to tumor progression and metastasis. In sum, we are beginning to connect the regulatory pathways to the mechanical networks that control cytokinesis shape change dynamics.

2361

The GEF/GAP Abr Differentially Regulates Rho and Cdc42 Activity Zones during Single Cell Wound Healing and Is Targeted by Binding to Active Rho.
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During X. laevis oocyte wound healing, organization of an acto-myosin contractile ring is controlled by zones of active Rho and Cdc42, which form concentric rings around wounds. We are now investigating how the activity of these GTPases is regulated during wound healing by GEFs, guanine nucleotide exchange factors, which activate the GTPases and GAPs, or GTPase activating proteins, which inactivate GTPases. To determine which GEFs and GAPs are involved in wound healing, we developed a functional screen based on effects on Rho and Cdc42 with overexpression, localization, and expression of a dominant negative construct. Of the GEFs and GAPs tested, only one, Abr, produced positive results in all 3 assays. Abr contains a GEF domain as well as a GAP domain, and has been shown In Vitro to act as a GEF for Rho, Cdc42, and Rac and a GAP for Cdc42 and Rac. Live imaging of GFP-Abr as well as immunofluorescence reveals localization of Abr to the trailing edge of the active Rho zone. Due to its localization, we hypothesized that Abr is recruited by binding to active Rho. As GAP domains bind to the active forms of the GTPases, we deleted the GAP domain and noted that this version of Abr can no longer localize to wounds. Similarly, the Rho inhibitor C3 abrogates Abr localization. Moreover, activation of Rho with GTPyS, expression of constitutively active Rho, and expression of a Rho GEF all promote localization of Abr to the plasma membrane. When overexpressed, Abr eliminates the zone of active Cdc42, but upregulates the zone of active Rho. Inhibition of Cdc42 activity was dependent on the GAP activity of Abr. Consistent with the positive regulation of Rho, when endogenous Abr is depleted using a morpholino approach in embryos, Rho activity is inhibited and healing is perturbed. These results suggest a mechanism whereby Abr could bind to and positively regulate Rho but inhibit Cdc42 in this same region, keeping the zones segregated. This work demonstrates a case for localization of a GEF/GAP via binding to a GTPase itself and also provides insight into crosstalk of GTPases.

2362

Cell-Substrate Adhesion Negatively Regulates Clathrin-Dependent Endocytosis.
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Clathrin-dependent endocytosis is a major method for the cellular import of macromolecules and occurs at the interface between the cell and its surroundings. However, little is known about the contribution of cell-substrate attachment to clathrin-coated vesicle formation. Here, we use biochemical and imaging-based methods to reveal that cell substrate adhesion reduces the rate of endocytosis. Clathrin structures in proximity to a subset of substrate contact sites form bright clusters and exhibit distinct dynamics from clathrin coated pits physically distant from the adhesion sites. By directly manipulating the extracellular matrix to modulate adhesion strength, we establish that tight adhesion dramatically reduces clathrin-dependent endocytosis of transferrin and extends the lifetimes of individual coated pits. This adhesion-mediated reduction in endocytosis is mediated through β1 integrin engagement with the matrix, and is controlled by the activity of focal adhesion kinase, a central regulator of focal adhesion signaling and turnover. In addition, through pharmacological perturbation of the actin cytoskeleton, we demonstrate that internalization of clathrin structures at sites of adhesion requires actin cytoskeletal dynamics. Together, our results demonstrate that cell-substrate adhesion strength regulates clathrin-dependent endocytosis, and that the actin cytoskeleton regulates clathrin dynamics at sites of adhesion.

2363

Sbf Scaffolds MTM Phosphatase and Class II PI3-Kinase to Coordinately Regulate PI(3)P-Mediated Roles in Endolysosomal Homeostasis and that Balance Cortical Dynamics Affecting Cell Shape.
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Cell compartmentalization is important for spatial control of cellular functions. The localized regulation of phosphoinositide phosphates (PIPs) distinguishes and controls membrane identities. Myotubularins (MTMs) encode for a large, conserved family of phosphatidylinositol 3-phosphate phosphatases, with half of the members encoding catalytically inactive or pseudophosphatases. Human mutations in active MTMR2 and pseudophosphatase MTMR13/Sbf2 are independently associated with Charcot-Marie Tooth neuropathy, and both proteins were shown to interact. However, little is known about the regulation and roles of the PIP dynamics and subpools impacted by coordinated MTM functions. We identified common roles for mtm and Sbf, the single Drosophila orthologs of MTM1/MTMR2 and MTMR5/MTMR13, respectively, through knockdown in fly blood cells, or hemocytes. Both mtm and Sbf were required in hemocytes for animal viability, PI(3)P turnover and the extension of dynamic cell protrusions. Conversely, overexpression of mtm or Sbf resulted in PI(3)P depletion and an increase number and extent of radial F-actin protrusions. Together, this suggests a novel PI(3)P-mediated mechanism both necessary and sufficient for modulating hemocyte morphology. One candidate effector, spire, with both predicted PIP-binding and F-actin regulatory functions, exhibited mtm-dependent PI(3)P localization and genetic interactions affecting cell protrusions. Unexpectedly, knockdown of Sbf did not phenocopy the mtm defect of enlarged endolysosomes, and co-RNAi depletion of Sbf and mtm rescued endolysosomal size. In this respect, Sbf function was similar to class II PI3K, PI3K68D, which we had shown to act antagonistic to all mtm-dependent functions. Bringing together this apparent lysosomal paradox, we found that Sbf scaffolds a protein complex through physical interactions with both Mtm and PI3K68D, as detected by co-immunoprecipitations from membrane fractions. Sbf may recruit Mtm and PI3K68D at distinct membrane compartments to coordinate the precise spatiotemporal control of PI(3)P, with important roles in endolysosomal homeostasis and in maintaining a dynamic balance of cortical regulators that affect cell shape.

Minisymposium 26: Cell Matrix Interactions and Signaling (2364 – 2369)

2364

Double-Edged Action of NEDD9 in Mammary Tumorigenesis.
The HEF1/CAS-L/NEDD9 scaffolding protein coordinates the FAK and SRC signaling cascades relevant to integrin-dependent adhesion, migration, and survival, and interacts with Rac to support mesenchymal movement in migrating cells. In the past 3 years, several studies have indicated elevated expression of NEDD9 contributes to cancer metastasis in a number of cancer types, while other work has suggested reduced NEDD9 expression is tumor-promoting. Our goal has been to dissect the mechanistic basis for NEDD9 actions in cancer development, and resolve these findings. Using two different mouse models for mammary tumorigenesis (MMTV-HER2 and MMTV-polymavirus T antigen, PyVT), show that the Nedd9 null genetic background significantly limits mammary tumor initiation. Action of Nedd9 is tumor cell intrinsic, with immune cell infiltration, stroma, and angiogenesis unaffected. The majority of the late-appearing mammary tumors of MMTV-PyVT;Nedd9-/- mice are characterized by depressed activation of proteins including SRC and FAK, but also AKT and ERK, emphasizing broad action of Nedd9 in support of pro-oncogenic signaling. Analysis of cells derived from primary Nedd9+/+ and Nedd9-/- tumors demonstrated persistently reduced FAK activation, attachment, and migration, consistent with a role for Nedd9 activation of FAK in promoting tumor aggressiveness. However, orthotopic re-injection or tail vein injection of cells derived from primary Nedd9+/+ and Nedd9-/- tumors revealed an unexpected reversal of aggressiveness profiles. Parallel In Vitro analyses indicated that absence of Nedd9 provided a strong selection for mutations that activated core proliferation-relevant signaling pathways by novel routes, while not affecting FAK/SRC signaling or cell migration controls. These latter activities likely reflect the additional interactions of Nedd9 with Aurora-A kinase, which regulate genomic stability. These results have significant implications for understanding the emergence of treatment-resistant breast tumors subsequent to targeted therapies.

Mechanotransduction during Axon Chemoattraction to Netrin.

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Axons are guided to their target by proteins distributed along their trajectory. These proteins can be transmembrane (e.g. ephrin-Bs and sema-1, -4, -5 & -6), chemically linked to the membrane (e.g. ephrin-As and sema-7) or secreted into the extracellular space (e.g. netrins, BMPs, sema-3 and slits). Although secreted, netrin-1 becomes attached to the extracellular space and we have found that it provides traction during axon chemotraction (1). Specifically, mouse spinal commissural neurons normally generate local pulls averaging 9 piconewtons, but will generate over 63 piconewtons directly on netrin-1. We are currently exploring the possibility that, in addition to providing traction, this mechanical interaction could stretch intracellular proteins in such a way that it influences intracellular signalling events. A particularly interesting candidate is p130CAS whose substrate domain becomes phosphorylated when stretched (2). We have confirmed that netrin-1 does indeed lead to increased Src-dependent phosphorylation of the substrate domain of p130CAS (3). I will present findings that directly test whether p130CAS functions as a mechanotransducers during chemoattraction to netrin-1. 1. S.W. Moore et al., Science 325, 166 (2009). 2. Y. Sawada et al., Cell 127, 1015 (2006). 3. G. Liu et al., J. Neurosci. 27, 957 (2007).
Cell polarization into anterior and posterior domains is essential for directional cell migration, associated with tissue morphogenesis and wound repair. Upon spreading on isotropic substrates, cultured fibroblasts demonstrate spontaneous polarization, progressing from a radially symmetrical to an elongated shape, with a single or a few lamellar protrusions comprising the leading edge. Characteristic features of polarized fibroblasts are stable concave edges, and long, straight actin bundles (stress fibers). Here, we show that the process of polarization is regulated by the cell’s adhesion-dependent mechanosensing of substrate rigidity. We plated fibroblasts onto polydimethylsiloxane or polyacrylamide substrates with rigidities ranging from several MPa (rigid), to 5 kPa (soft). The substrates were coated with either fibronectin or collagen, prior to cell plating. We found that cell spreading and the formation of focal adhesions were not disrupted on the soft substrate; yet cell polarization was dramatically suppressed on the soft matrix. Furthermore, focal adhesions that formed on the soft matrix were significantly smaller, albeit more numerous, than those that formed on the rigid substrate. Deprivation of integrin signaling by coating the substrates with polylysine instead of fibronectin or collagen, abolished the ability of the cells to distinguish between soft and rigid substrates. Similarly, treatment with blebbistatin, an inhibitor of actomyosin contractility, caused the cells spreading on the substrate to form similar shapes, regardless of substrate rigidity. To explore novel signaling pathways involved in rigidity sensing, we performed a high-throughput, high-resolution, microscopy-based screen, using siRNAs targeting 85 human protein tyrosine kinases (PTKs). We found that selective PTK depletion induced several characteristic phenotypes. Knockdown of some PTKs rescued the ability of the cells to polarize on the soft substrate, while knockdown of others reduced polarization on the rigid substrate. Suppression of a third group of kinases enhanced the cells’ differential responses to soft and rigid substrates. Thus, multiple tyrosine kinase-mediated pathways control rigidity sensing-driven cell polarization.

2367
Rapping Up Cell Adhesion.
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Rap1 GTPases regulate cell-matrix and cell-cell adhesion by interacting with specific Rap effectors. Cell-directed increases in Integrin affinity (“activation”) controls cell migration, matrix assembly, leukocyte trafficking, and hemostasis. Talin binding to the integrin β cytoplasmic domain is a final step in activation. Rap1 induces the activation of integrins by binding to RIAM, the product of the APBB1IP gene, to promote talin-dependent integrin activation. We find that that RIAM connects the membrane targeting sequences in Ras GTPases to talin, thereby recruiting talin to the plasma membrane and activating integrins. To prove this, we created a minimized 50 residue Rap-RIAM module containing the talin binding site of RIAM joined to the membrane-targeting sequence of Rap1A that targeted talin to the plasma membrane and mediated integrin activation. KRIT1 is a Rap effector and genetic deletions of KRIT1, CCM2, or CCM3 in humans and other vertebrates produce similar cardiovascular phenotypes. KRIT1 functions as a scaffold by physically interacting with CCM2 and thus CCM3 participates in multiprotein complexes containing Rho GTPases and numerous kinases and phosphatases. The genetic and biochemical relationships of KRIT1 and CCM2-3 indicate that the assembly and regulation of these multiprotein complexes is an important regulator of cardiovascular development. We found that KRIT1 localizes to endothelial and epithelial cell junctions and controls their integrity. Rap1 regulated the junctional localization of KRIT1, which is mediated by the interaction of KRIT1 with the transmembrane receptor, heart of glass (Heg1) and previous work showed that deletion of Heg1 produced a similar phenotype to loss of KRIT1 in zebrafish. These KRIT1 multiprotein complexes act through Rho GTPases to control the cytoskeleton and thus, junctional integrity.

2368
Tumor Inflammation and Progression Depend on PI3-Kinase-γ-Mediated Activation of Integrin α4β1 In Myeloid Cells.
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Cancer and inflammation are linked, as chronic inflammatory diseases increase the risk of developing tumors, and growing tumors induce host inflammatory responses that stimulate tumor progression. Myeloid cells, including granulocytes, monocytes, myeloid-derived suppressor cells and tumor-associated macrophages invade the tumor microenvironment in response to a variety of chemoattractants and promote tumour angiogenesis, immnosuppression or metastasis. The mechanism by which these chemoattractants stimulate myeloid cell accumulation is incompletely understood. Here, we show here that a single, common Ras-PI3-kinase- integrin α4β1 pathway regulates myeloid cell extravasation, tumor inflammation and progression, regardless of the specific chemoattractant produced by the tumor. Chemoattractants released from tumor cells, including SDF-1α, TNFα and VEGF-A, and from tumor macrophages, such as IL-1β and IL-6, stimulate Ras, PI3-kinase and Rap1-dependent integrin α4β1 activation and integrin α4β1-dependent recruitment of myeloid cells to the tumor microenvironment. Genetic or chemical inhibition of N- and K- Ras, PI3-kinase, Rap1 or integrin α4β1 blocks myeloid cell adhesion to vascular endothelium and recruitment to implanted or spontaneous tumors, leading to a reduction in tumor angiogenesis, growth and metastasis. These findings help to define further the role that myeloid cells play in cancer and indicate for the first time that use of inhibitors of PI3-kinase or integrin α4β1 represents an innovative approach to control tumor malignancy.

ARF6-Regulated Shedding of Plasma Membrane-Derived Microvesicles Facilitates Cell-Matrix Interactions and Matrix Degradation.

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Increased MAPK signaling, small GTPase activation, cytoskeletal rearrangements and the directed targeting of proteases to sites of extracellular matrix (ECM) degradation, all accompany the process of tumor cell invasion. Several studies have implicated the small GTP-binding protein, ARF6, in tumor cell invasion although the molecular basis by which ARF6 facilitates this process is still unclear. Here we show that ARF6 activation regulates the release of plasma membrane-derived, protease-loaded, membrane microvesicles from tumor cells into the surrounding environment to promote ECM degradation. These ‘invasive’ microvesicles are distinct from invadopodia, which also mediate cell invasion. To enable microvesicle shedding, ARF6-dependent activation of phospholipase D promotes the recruitment of the extracellular signal-regulated kinase (ERK) to the plasma membrane where in turn, ERK phosphorolyses and activates myosin light chain kinase (MLCK). MLCK-mediated MLC phosphorylation is required for microvesicle release. Protein cargo appears to be selectively sorted into microvesicles and adhesion to the ECM is facilitated by vesicle-associated beta-integrin receptors. We propose that microvesicle shedding is designed to release selected cellular components into the surrounding environment, particularly those involved in cell-matrix interactions and cell motility. These findings are significant in light of recent reports that ARF6 activation as well as proteolytic activities of microvesicles shed by tumor cells correlate directly with invasiveness and tumor progression.

Minisymposium 27: Cell Senescence and Cell Death (2370 – 2375)

Using Knock-in Mice to Define p53’s Mechanism of Action in Senescence, Apoptosis, and Tumor Suppression.
The crucial role of the p53 protein in tumor suppression is underscored by the findings that p53 is mutated in over half of all human cancers and that p53-deficient mice universally develop cancer. While p53 suppresses cancer by inducing cell cycle arrest, senescence, or apoptosis in response to stress signals, its molecular mechanism of action In Vivo remains unclear. p53 displays activity as a transcriptional activator that can induce numerous target genes, but it also has other biochemical functions, including transcriptional repression and induction of mitochondrial membrane permeabilization. To define the contribution of transactivation for p53 function in vivo, we generated knock-in mouse strains expressing different transactivation-defective mutants. In our initial experiments, we analyzed the properties of p53-25,26, a mutant with alterations in the first of two p53 transactivation domains. We showed that p53-25,26 is greatly compromised for transactivation of many p53 target genes, but retains the ability to activate a small subset of p53 target genes. Analysis of the biological activity of p53-25,26 revealed that it exhibits a clear selectivity in its activities. Although unable to induce growth arrest or apoptosis in response to acute DNA damage signals, it can trigger responses downstream of oncogenic signals, including senescence and tumor suppression. These data suggest that full transactivation potential is not essential for p53 tumor suppressor function, and that instead, either very minimal transactivation or another activity of p53 altogether is important for its function. To distinguish these possibilities, we have generated and are analyzing knock-in mice expressing a p53 mutant with alterations in both the first and second transactivation domains, p53-25,26,53,54. Analyses of these mutant mice will provide a powerful means for better defining the functions of p53 critical for tumor suppression in different settings.

2371

In Neurons, the Checkpoint Kinase ATM Protects Against Reactive Oxygen Induced Cyclin D1 Elevation and Cell Death.

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Our past studies suggest that regulation of cyclin D1 by ATM might play a role in protection against neural degeneration. Ataxia-telangiectasia results from mutation of the ATM gene. The associated ataxia is caused by progressive neuronal cell death, which in other systems has been related to increased cyclin D1 expression leading to aberrant cell cycle entry. The challenge has been to explain how this can be caused by loss of the ATM checkpoint kinase. A possible clue to this question was suggested by our recent work indicating that a decline in cyclin D1 levels during S phase is essential for normal cell cycle progression, and that this decline can be controlled by the checkpoint kinase ATR. The related kinase, ATM, was similarly found to suppress cyclin D1 levels following DNA damage. Because ATM is able to control cyclin D1 expression, and because cyclin D1 elevation can induce cell death in post-mitotic neurons, we initiated studies to test the connection between ATM and cyclin D1 in neural cells. We specifically tested the possibility that ATM and cyclin D1 play a critical role in the survival of neurons following exposure to oxidative stress. As a model system, we utilized immortalized human neural stem cells (from Millipore) which can be induced to differentiate into neurons following growth factor deprivation. In mature neurons where cyclin D1 levels are naturally low, treatment with hydrogen peroxide together with inhibition of ATM resulted in the stimulation of cyclin D1 levels. The inhibition of ATM activity (with the specific inhibitor KU55933) also dramatically increased neuronal cell death following peroxide treatment. Importantly, the dying neurons in these cultures (with the neuronal marker betaIII tubulin and condensed chromatin) were commonly found to have high levels of cyclin D1 in their nuclei. We conclude that the DNA damage checkpoint kinase ATM potentially plays a critical role in regulating cyclin D1 levels and death of neuronal cells following exposure to oxidative stress. This might help explain the neural effects of Ataxia-telangiectasia, and increase our understanding of neuronal cell death in general.
2372
SACK-Expansion of Human Hepatocytic Adult Stem Cells That Display Immortal DNA Strand Co-Segregation.
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There is clinical interest in expanding human liver adult stem cells for the purposes of gene therapy, tissue engineering, drug development, and cell replacement therapies. Previously, it has been difficult to expand pure clonal populations of human liver stem cells. In Vivo and ex vivo, adult stem cells divide with asymmetric cell kinetics. In addition to their special cell kinetics, when adult stem cells asymmetrically self-renew, they segregate their chromosomes non-randomly. By retaining a set of template DNA strands (called “immortal DNA strands”), they avoid carcinogenic mutations from replication errors. In previous studies with adult rat liver cells, we established that asymmetric cell kinetics is a major barrier to expansion of adult stem cells in culture. We have addressed this problem with a strategy developed in our lab called suppression of asymmetric cell kinetics (SACK). By shifting their cell kinetics pattern from asymmetric to symmetric, the exponential expansion of adult stem cells can be promoted. Here, we report the adaption of the SACK method to expand human liver adult stem cells ex vivo. As an improvement to restrict the growth of non-stem cells, we reduced the serum concentration and added EGF and TGF-beta. With this modified SACK method it was possible to consistently establish post-natal human liver adult stem cell cultures and establish clonal strains from them. We were able to maintain these SACK-derived cultures without senescence over 80 cell population doublings. Characterization of the established strains show evidence of asymmetric cell kinetics by time-lapse, SACK dependent proliferation, ability to produce cells with mature hepatocyte properties and secrete hepatocyte-specific extracellular matrix proteins. In addition, these strains show first evidence of human cells with immortal DNA strand co-segregation by both BrdU label-retention studies and BrdU label-exclusion studies.

2373
Mst1 Is an Interacting Protein That Mediates Phlpps Induced Apoptosis in Cancer Cells.
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PHLPP and PHLPP2 phosphatases exert their tumor suppressing functions by dephosphorylation and inactivation of Akt in several breast cancer and glioblastoma cells. However, Akt, or other targets of PHLPPs including PKC and ERK, may not fully account for the physiological roles of these multifunctional phosphatases. Here we demonstrate that PHLPPs induce apoptosis in cancer cells independent of the known targets of PHLPPs. An unbiased screen for novel pathways identified Mst1 as a binding partner that interacts with PHLPPs both In Vivo and In Vitro. PHLPPs dephosphorylate Mst1 on the T387 inhibitory site resulting in activation of Mst1 and its downstream effectors p38 and JNK to induce apoptosis. The same T387 site can be phosphorylated by Akt. Thus, PHLPP, Akt and Mst1 create an auto-inhibitory triangle that controls the fine balance of apoptosis and proliferation in a cell type and context dependent manner.

2374
Calcium Signal Is Involved in the Cadmium-Induced Generation of Reactive Oxygen Species and Activation of MAPK/mTOR Network Leading to Neuronal Cell Death.
Cadmium (Cd), an environmental pollutant, has been demonstrated to contribute to neurodegenerative disorders. However, the molecular mechanism of Cd-induced neuronal cell damage is still not clear. Recently we have shown that Cd induces reactive oxygen species (ROS) and activates MAPK/mTOR network, leading to neuronal apoptosis. To further unveil the underlying mechanism, here we studied whether calcium signaling is involved in these events with Cell Viability assay, ROS generation assay and Western blot analysis. Treatment with EGTA (extracellular calcium chelator), BAPTA-AM (intracellular calcium chelator) and trifluoperazine dihydrochloride (TFP) (calmodulin inhibitor) significantly blocked Cd-induced cell death and ROS generation in PC12 and SH-SY5Y cells. Furthermore, EGTA, BAPTA-AM and TFP inhibited Cd-induced phosphorylation of the extracellular signal-regulated kinase 1/2 (Erk1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK. Also, Cd-triggered a canonical signaling cascade that is PI3K, PDK1, Akt, TSC2, Rheb, mTOR, S6K1, S6 and 4EBP1 was abolished by inhibition of calcium/camodulin signal with these compounds. Inhibition of Akt phosphorylation in Thr308 and Ser473 mimicked the effect of the calcium chelators or the calmodulin inhibitor, reducing Cd-induced ROS generation and neuronal cell death. Moreover, Akt inhibitor decreased Cd-activated MAPK and mTOR pathway. Finally we identified that blocking calcium signal and Akt inhibitor both suppressed Cd-induced up-regulation of NADPH oxidase family members including NOX2, p22phox, p40phox, p47phox and p67phox. In conclusion, calcium/camodulin signal is essential for Cd-induced ROS generation and MAPK/mTOR activation associated to neuronal cell death.

2375
Metabolism Meets Apoptosis: A Role for Pro-Apoptotic BAD in Glucose Sensing.
N. Danial; Department of Pathology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA

Cellular energy homeostasis requires careful orchestration of ATP production and utilization. Glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) are the major cellular ATP generating pathways and their utilization is controlled by the availability of nutrients and oxygen. Improper shift in the utilization of ATP generating pathways results in metabolic aberrations. As such, suppression of mitochondrial oxidative capacity is associated with both cancer and diabetes. Identification and characterization of molecules that govern the cell's choice of ATP regenerating systems will provide important insights into targeting energy metabolism in disease. We have previously shown a novel role for the BCL-2 family protein BAD in glucose oxidation. Recent studies indicate that the BH3 domain of BAD, previously known as its minimal death domain, is required and sufficient for glucose oxidation. Importantly, phosphorylation of a defined residue within this domain constitutes a physiologic switch between its metabolic and apoptotic functions. When phosphorylated, BAD BH3 domain engages a metabolic program that includes glucose phosphorylation, mitochondrial respiration and ATP production. We further highlight the pharmacologic relevance of phosphorylated BAD BH3 domain by demonstrating the metabolic activity of hydrocarbon-stapled BAD BH3 helices. Our studies define an alternative target and function for the BAD BH3 domain and emphasize the therapeutic advantage of its mimetics. The significance of BAD's dual functionalities in different cell types, including pancreatic beta cells, will be discussed.

Minisymposium 28: ES Cells, iPS Cells, and Germ Cells (2376 – 2381)

2376
Cell Fate Decisions in the Human Embryo.
Human embryo development begins with the fusion of egg and sperm, a remodeling of the maternal and paternal pronuclei and a series of cleavage divisions. Subsequently, on Day 3, the embryonic genome is activated and the stage is set for a series of cell fate decisions that lead to formation of the distinct tissues of the blastocyst, the primary germ layers and the germ cell lineage. Our recent findings indicate that human embryo development is characterized by a complex pattern of gene expression with the vast majority of genes that are modulated being down-regulated. Moreover, we observed that the majority of genes that are expressed in early human preimplantation development are of unknown function/identity. Thus, to probe function, we developed the tools necessary to examine one of the earliest decisions in human embryo development, namely the allocation of cells to the germ cell lineage. We have found that both human embryonic stem cells and human adult- and fetal-derived induced pluripotent stem cells can form both primordial germ cells and meiotic germ cells. Moreover, the use of hESCs and iPSCs allows the genetic dissection of human germ cell and somatic cell formation; we have found that a family of translational factors regulates germ cell formation, maintenance and differentiation.

**2377**

**A C. elegans LSD1 Demethylase Contributes to Germline Immortality by Reprogramming Epigenetic Memory.**

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Extensive epigenetic reprogramming occurs during cloning and in the induction of pluripotent stem cells (IPS). Similarly, during normal germ line transmission, the epigenetic state of the genome is known to undergo extensive reprogramming both in the gametes and in the early embryo. This reprogramming may be essential to re-establish a developmental ground state between generations, but the mechanism of these epigenetic reprogramming events remains poorly understood. We show that mutants in spr-5, the C. elegans ortholog of the H3K4me2 demethylase LSD1/KDM1, exhibit progressive sterility over many generations. This sterility correlates with misregulation of spermatogenesis-expressed genes and transgenerational accumulation of the histone modification dimethylation of histone H3 on lysine 4 (H3K4me2). This suggests that H3K4me2 can serve as a stable epigenetic memory and that erasure of H3K4me2 by LSD1/KDM1 in the germline prevents the inappropriate transmission of this epigenetic memory from one generation to the next. Thus, we propose that the demethylation of H3K4me2 by LSD1/KDM1 comprises part of the epigenetic reprogramming between generations. We also hypothesize that LSD1/KDM1 demethylation may be part of the somatic reprogramming that is induced during the generation of IPS cells. To pursue the role of LSD1/KDM1 in epigenetic reprogramming further, we have recently generated mice with a germline deletion of LSD1/KDM1. The preliminary results from these ongoing experiments will be discussed.

**2378**

**Dual Roles of Stem Cell Antigen-1 Regulation in the Bone Marrow.**

H. C. Kwan; CVRI, UCSF, San Francisco, CA

Dual Roles of Stem Cell Antigen-1 Regulation in the Bone Marrow Helen C. K. Kwan, Saami Khalifian, Carissa Ritner, Walter J. Liszewski, Harold S. Bernstein Cardiovascular Research Institute, Department of Pediatrics and Eli and Edythe Broad Center of Regeneration Medicine & Stem Cell Research University of California San Francisco, San Francisco, California ABSTRACT Stem cell antigen-1 (Sca-1; Ly6A) is a GPI-anchored protein widely used as a cell surface marker for tissue-specific stem cells such as hematopoietic, mammary, prostate, retinal, and skeletal muscles. Our laboratory identified Sca-1 as a marker of activated myoblasts, and subsequently
demonstrated both In Vitro and In Vivo that Sca-1 regulates myoblast proliferation, suggesting an important function for Sca-1 in skeletal muscle homeostasis, and a potential role in muscle stem cell maintenance. Since Sca-1 influences the tempo of myoblast proliferation following injury, we hypothesized that it may also affect stem cell activation and self-renewal. To study this, we adopted the well-studied hematopoiesis system as a model and injected mice with 5-fluorouracil (5FU) to stimulate bone marrow (BM) "injury" and hematopoietic stem cell (HSC) activation. The Lin- c-kit+ and Lin- c-kit- subpopulations both increased 6-fold in Sca-1+/+ bone marrow 72 hrs post-injection, demonstrated HSC activation by 5-FU. Injured Sca-1-/- bone marrow, however, demonstrated a 6-fold increase in Lin- c-kit+ cells but a lesser increase (3-fold) in the Lin- c-kit-subpopulation at the same time point. Expression profiling of bone marrow cells from Sca-1 null injured mice versus their null siblings demonstrated an up-regulation of Mpl, Myct1, Tgfb1, Jak3, and many other known regulators in self-renewal pathways or cellular proliferation. Down regulation of Pten was observed expectedly. However, Pten was upregulated in Sca-1 null animals versus wild-type siblings. Interestingly, an increase of Lin-c-kit+ subpopulation was found in Sca-1 null animals versus wild type. Additional studies of LT-HSC versus its derivatives in these animal subgroups will dissect out the roles of Sca-1 in HSC activation.

2379
Development of Platform and Assays for High Throughput Analysis of Cardiomyocytes Physiology and Toxicity.
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Cardiomyocytes are contractile cells characterized by complex physiology and are often the main target of the toxic effect of natural compounds and drugs. We describe the development of instrumentation and assays to analyze parameters relevant to cardiomyocytes activity in cell culture systems. An optical method has been developed to stimulate cardiomyocytes contraction by light. An automatic image analyzer has been designed to record and quantify single cells calcium flux, ATP levels, apoptosis and virtually any parameter measurable by luminous readout. The instrument and assays have been applied to neonatal rat ventricular cardiomyocytes (NRVCs) and stem cell-derived cardiomyocytes. As examples of potential applications we have quantified the prolongation of calcium flux in cells where the expression of the SERCA2 calcium pump was inhibited by siRNA. We have also measured single cell calcium transient, ATP levels and apoptosis in samples treated with well-known cardiotoxic compounds. Our results validate the platform and assays and imply the possibility of conducting high throughput multi-parameter analysis in living cardiomyocytes.

2380
Regulation of Asymmetric Stem Cell Division in the Drosophila Male Germ Line.
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Adult stem cells are the source of continuous supply of differentiated cells, thus maintaining tissue homeostasis throughout the life of organisms. Upon stem cell division, its daughters must decide between self-renewal and differentiation, a failure of which can lead to tissue hyperplasia or degeneration. To maintain this critical balance, many stem cells have a potential to divide asymmetrically, giving rise to one stem cell and one differentiating cell. Drosophila male germ line stem cells (GSCs) divide always asymmetrically, producing one self-renewed stem cell and one differentiating cell. This asymmetric stem cell division is tightly controlled via stereotypical positioning of centrosomes, which leads to oriented spindle with respect to the stem cell niche and thus asymmetric stem cell division. Recently, we have shown that GSCs with "misoriented centrosomes" (i.e. stereotypical centrosome positioning is disrupted) are delayed/arrested in the
cell cycle, implying the presence of a novel checkpoint to monitor centrosome orientation prior to mitosis, ensuring asymmetric outcome of the stem cell division. Here we show that Par1 kinase is the component of this checkpoint. In GSCs mutant for Par1, GSCs undergo mitosis irrespective of centrosome orientation, leading to high frequency of misoriented spindles. Overall rate of stem cell division must be modulated to accommodate to the change in the availability of nutrients; under a condition with limited availability of nutrients, organisms must slow down the overall metabolism and proliferation of cells. We found that centrosome misorientation of GSCs significantly increase in the flies cultured in a poor-nutrient media, thus slowing down overall stem cell division, presumably via activation of centrosome orientation checkpoint. This response is dependent on the insulin signaling pathway. We propose that insulin signaling controls centrosome orientation to regulate the rate of stem cell division, depending on nutrient availability.

2381
Human Pluripotent Stem Cell Models of Alzheimer's Disease and Niemann-Pick Type C.
L. Goldstein; Department of Cellular and Molecular Medicine, University of California, San Diego School of Medicine/HHMI, La Jolla, CA

Neurons are large highly polarized cells that depend upon long-distance microtubule-based transport between synapses and cell bodies to support signaling and neurotransmission. In previous work, we used fruit flies, mice, and humans to study the nature of molecules that link molecular motor proteins to vesicular cargoes in axons and discovered close relationships between these molecules, signaling, and neurodegenerative diseases. We are now using human embryonic stem cells to develop new systems for testing these ideas and for understanding, and eventually treating Alzheimer's Disease and a related disease, Niemann-Pick Type C (NPC). for example, we are generating human neurons from human IPS cells that carry familial Alzheimer's Disease mutants to try and test several hypotheses of disease causation. Similarly, we are using human neurons generated from human embryonic stem cells in which the expression of the NPC1 gene has been knocked down. Finally, because the relationship of familial forms of Alzheimer's Disease to the common "sporadic" form of the disease remains unclear, we are analyzing human neurons differentiated from IPS cells made from patients with sporadic forms of AD. Specifically, biochemical and cellular comparisons of defined types of human neurons either carrying familial mutations, or carrying genomes from patients with sporadic AD may yield an understanding of what components of sporadic disease are defined by genetic characteristics.
Minisymposium 29: Molecular Motors (2382 – 2387)

2382
Optimization of Kinesin-13 Microtubule Depolymerase Activity Reveals a General Mechanism for Diffusional Motility.
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The kinesin-13, MCAK, is a critical regulator of microtubule dynamics in cells. Using mutational analysis we have functionally dissected the structural features that make MCAK the most potent microtubule depolymerizer observed to date. MCAK’s positively charged neck enhances delivery to microtubule ends, not by tethering the molecule to microtubules during diffusion, as is commonly thought, but by simultaneously increasing both the association and dissociation rate of the molecule on to the microtubule. Counterintuitively, it is this feature that enables MCAK to rapidly target microtubule ends. While the positively charged neck greatly enhances MCAK’s delivery to microtubule ends and also its global depolymerization efficiency, the neck slightly diminishes MCAK’s ability to remove tubulin subunits once at the end. Conversely, dimerization reduces MCAK’s delivery to ends but improves MCAK’s ability to remove tubulin subunits. The observed binding kinetics for these reactions predict a novel non-specific binding mechanism that represents a paradigm for the diffusive interaction of many microtubule binding proteins.

2383
Tail Domain-dependent Regulation of the Kinesin-8 Depolymerase.
X. Su1,2, M. Gupta3, W. Qiu4, S. Reck-Peterson5, D. Pellman1,2; 1Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, 2Howard Hughes Medical Institute, Harvard Medical School, Boston, MA, 3Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL, 4Department of Cell Biology, Harvard Medical School, Boston, MA

Kinesin-8s are unique among the kinesin-superfamily of proteins because they possess both plus end-directed microtubule motility and microtubule depolymerase activity. Kip3, the budding yeast kinesin-8 motor, regulates microtubule dynamics and is involved in processes such as spindle positioning, chromosome segregation and spindle disassembly. Similar to conventional kinesin, Kip3 contains an N-terminal motor domain, an internal coiled-coil region, and a C-terminal ‘tail’ domain, the function of which is unknown. Here we report that the C-terminal tail domain of Kip3 negatively regulates Kip3’s activity through a mechanism that involves tubulin dimer binding. Kip3 truncations lacking the tail domain are hyperactive in vivo. Microtubules show increased shrinkage rate and more frequent catastrophes in cells expressing the tail-deleted mutant. However, in vitro, the recombinant tail-less Kip3 was similar to the full-length motor for ATPase activity, motility, and depolymerase activity. Interestingly, a difference between the tail truncation and the full-length motor was revealed in the presence of free tubulin dimers: tubulin dimers inhibited the depolymerase activity and apparent on-rate of full-length Kip3 but to a lesser extent than the tail truncation. The tail truncation bound free tubulin less efficiently than full-length Kip3. Taken together, these data suggests the tail domain facilitates the binding of Kip3 to tubulin dimers; dimer binding sequesters soluble Kip3, decreasing its effective concentration. The tail-facilitated sequestration effect appears to be relevant in cells, because the tail truncation increased Kip3 microtubule association in vivo. The potential implications of these results for the general regulatory mechanism of kinesin-8 motors will be discussed.

2384
Novel Role for Microtubule Motor Protein Eg5 in Protein Translation.
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Kinesin motor proteins are important for the transport of specific intracellular cargo along microtubules. In addition to this transport function, some kinesins have been implicated in mitosis and are critical for proper cell division. Eg5 is a plus-end directed microtubule motor that localizes to spindles during mitosis. To date, the characterization of mitotic motor Eg5 has been almost exclusively limited to mitosis. However, unlike other mitotic regulators, mitotic motor proteins are expressed robustly regardless of the phase of the cell cycle. Such constitutive expression led us to investigate potential interphase functions of these proteins. Through ribosomal subunit fractionation studies and immunoprecipitation, we have determined that during interphase Eg5 predominantly associates with ribosomes. In addition, inhibition of Eg5 function, either by a knockdown or a small molecule inhibitor, resulted in a marked decrease of 40-60% in protein translation. Through polysome profiling studies, we have determined that after knockdown or inhibition of Eg5, the decrease in protein translation is accompanied by an increase in the 80S complex, which suggests a defect in translation elongation. Further confirmation of this defect was observed by completing ribosome transit assays, which refer to the time it takes one ribosome to transverse across a single mRNA, and demonstrated that after Eg5 inhibition by Monastrol, the ribosomal half-transit time increased from 91.2 sec in control cells to 130.4 sec after Eg5 inhibition. Furthermore, through In Vitro microtubule binding assays, we observed that the 80S ribosome binds microtubules via Eg5 in mammalian cells and in addition, microtubules are also required for translation, as inhibition of microtubules by two different microtubule inhibitors resulted in decreased protein translation. Collectively, our data proposes that Eg5’s major function in a cell is to aid protein translation and suggests a novel role for mitotic microtubule motor Eg5 in protein translation.

2385
Coupled Myosin VI Motors Facilitate 10 μm Long Unidirectional Movement on a Dense Dendritic F-Actin Meshwork.
S. Sivaramakrishnan, J. A. Spudich; Stanford University, Stanford, CA

Unconventional myosins interact with the dense cortical F-actin meshwork during processes such as membrane trafficking, cell migration and mechanotransduction. Our understanding of unconventional myosin function is derived largely from assays that examine the interaction of a single myosin with a single F-actin filament. In this study we have developed an In Vitro motility assay to study the interaction between multiple tethered unconventional myosins and a model dendritic F-actin cortex, namely the lamellipodium of a migrating fish epidermal keratocyte. Using myosin VI, which moves towards the minus-end of actin filaments, we directly determine the polarity of the entire keratocyte lamellipodium. We use a combination of experiment and simulation to demonstrate that multiple myosin VI molecules can coordinate to efficiently transport vesicle-size cargo over 10 μm of dense dendritic F-actin meshwork. Our assay reveals that unconventional myosins, which are monomeric and hence non-processive in single molecule assays, can transport cargo regardless of the oligomerization state of the myosin on the cargo.

2386
Regulation of Cytoplasmic Dynein Motor Activity.
S. Reck-Peterson¹, J. Huang¹, J. Kardon², C. Cho², R. Vale²; ¹Department of Cell Biology, Harvard Medical School, Boston, MA, ²Cellular and Molecular Pharmacology, UCSF School of Medicine, San Francisco, CA

Cytoplasmic dynein is a microtubule-based molecular motor responsible for nearly all minus-end directed microtubule based transport in cells. We use the model system S. cerevisiae to purify recombinant cytoplasmic dynein and its associated complexes and subunits (dynactin, lis1 and nudel). Using this recombinant system we are able to easily engineer point mutations, truncations and genetic tags that allow us to add a variety of handles that can be used for purification or fluorescent labeling. We then measure the activity of these complexes using a number of assays including single molecule fluorescence microscopy. Our results reveal that there are a variety of mechanisms that can be used to regulate the motor activity of cytoplasmic dynein.
A Neurodegenerative Mutation in Cytoplasmic Dynein Reveals Novel Regulation of Motor Activity.

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Cytoplasmic dynein is a minus-end directed microtubule motor protein that is responsible for many cellular functions including fast retrograde axonal transport. Two mouse strains (Loa and Cra1) have been reported with mutations in the dynein heavy chain gene that impair transport. Of equal interest, mice from each strain undergo progressive lower motor neuron degeneration (MND) similar to ALS. Because axonal transport defects could contribute to MND, we present a detailed study of the biochemical and biophysical properties of one of the mutant forms of dynein to understand how dynein function might be impaired. The Loa mutation is located within the tail domain of the dynein heavy chain, a region implicated in dynein intermediate chain binding, and heavy chain dimerization. We found the mutant dynein complex to be less stable than the wild type complex as judged by sucrose density centrifugation of brain cytosol, a difference further exaggerated by the addition of potassium iodide. To test how altered stability might affect dynein function, we purified the motor protein from stockpiled brains of wild type and Loa/+ mice. Subunit composition was virtually identical for wild type and Loa/+ dynein. Although basal ATPase activity was the same for both preparations of dynein, the mutant dynein exhibited a dramatic increase in the Km, but a normal Vmax, suggesting a decreased affinity for microtubules. This was confirmed by microtubule binding assays, which revealed decreased affinity of the mutant dynein in the presence, though not the absence, of ATP. We performed a complete characterization of wild type and mutant dynein at the single molecule level, including processivity and force measurements, using a combination of optical trap and quantum-dot based assays under a variety of conditions. Compared with wild type dynein, the mutant dynein exhibited a 23% to 50% reduction in run length. These results provide the first indication that dynein tail mutations can affect motor activity. By impairing dynein’s processivity, the Loa mutation likely compromises the ability of mutant dynein to efficiently transport cargo along the axon, causing MND in mice.

Minisymposium 30: RNA Biology (2388 – 2393)

Single Cell mRNA Analysis: RNA drives Cellular Phenotype and Functionality of mRNA containing Retained Introns in Neurons.

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Single cell mRNA analysis has provided insight into the complexities of gene expression and regulation that are difficult to obtain from mixed cell populations. For example, in neurons there are a large number of different cytoplasmic RNAs that have retained introns. These RNAs do not undergo NMD. These retained introns serve multiple functional roles in the cell from 1) directing the subcellular targeting of mRNAs, 2) targeting the site of protein expression and 3) regulation of cytoplasmic exon utilization. These intron-retaining RNAs and their canonically spliced sibs provide the RNA that constitute the expression profile of a cell. The expression profile is the relative abundances of mRNAs within a cell. Such expression profiles are diagnostic of cell type, physiological state of the cell and disease state. The role of these mRNA populations in driving and maintaining cellular phenotype will be discussed. This discovery was based upon the use of the Transcriptome Induced Phenotype Remodeling (TIPeR) paradigm that was recently established.
2389
Phosphorylation of Tristetraprolin (TTP) by the p38-Activated Kinase MK2 Impairs Deadenylase Recruitment and mRNA Decay.
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Human cells can fine-tune gene expression in response to a rapidly changing cellular environment by regulating the rates of mRNA decay. Several cell signaling pathways are known to modulate mRNA decay, but it is poorly understood how these signals are communicated to the mRNA decay machinery. Tristetraprolin (TTP) triggers the rapid decay of a class of transiently expressed transcripts encoding cytokines, growth factors and proto-oncogenes by binding AU-rich elements (AREs) encoded within 3' UTRs and recruiting enzymes involved in mRNA turnover. The ability of TTP to activate mRNA decay is impaired upon phosphorylation by the p38 MAPK-activated kinase, MK2. The mechanism behind the suppression of TTP as a result of phosphorylation is currently unknown. To understand the mechanism by which cell signaling events regulate mRNA decay, we examined the ability of phosphorylated and non-phosphorylated TTP to bind ARE-mRNA and to recruit mRNA decay enzymes, as well as its ability to activate mRNA decay in cells. We observed that phosphorylation of TTP does not appear to impair mRNA binding in co-immunoprecipitation assays. However, phosphorylated TTP does show a markedly reduced association with the major cytoplasmic deadenylases. In addition, the rate of deadenylation of an ARE-mRNA is reduced in cells expressing phosphorylated TTP relative to those expressing non-phosphorylated TTP. In contrast, phosphorylation of TTP does not appear to impair its ability to activate decapping of mRNA. These results indicate that phosphorylation of TTP primarily affects mRNA decay downstream of RNA binding through the failure to recruit the deadenylation machinery. Thus, TTP may remain poised to rapidly reactivate the deadenylation of bound transcripts in order to down-regulate gene expression upon the deactivation of the p38 MAPK pathway.

2390
Functional Importance of the di-sRNP Structure of Archael Methylation-guide Box C/D sRNPs.
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Methylation of ribosomal RNA (rRNA) is required for optimal protein synthesis. Multiple 2'-O-ribose methylations are carried out by small (nucleolar) box C/D guide ribonucleoproteins (s(no)RNPs), which are ubiquitous in nature from archaea to eukaryotes. Each site of methylation is dictated by base pairing between the specific guide s(no)RNA component of the s(no)RNP and the target rRNA. We have determined the first structure of a reconstituted and catalytically active box C/D sRNP by electron microscopy and single particle analysis. Our results reveal that archaeal box C/D sRNPs form an unexpected di-sRNP structure, challenging the conventional view of box C/D s(no)RNP architecture. Analysis of mutations in conserved regions of protein components as well as in the sRNA components of the RNP by several biochemical methods indicates that efficient formation of the di-sRNP is important for enzymatic activity of the archaeal methylation-guide RNPs.

2391
A Cajal Body Is not Essential for Post-Transcriptional Modification of Spliceosomal snRNAs.
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Cajal bodies (CBs) are small nuclear organelles found in a wide variety of eukaryotic organisms, usually identified by the marker protein coilin. The composition of CBs suggests that they are involved in assembly and maturation of the RNA processing machinery of the nucleus. In mammalian cells it has been shown that modification of splicing snRNAs, both 2'-O-methylation and pseudouridylation, normally takes place in the CB. These modifications are mediated by small CB-specific RNAs (scaRNAs). However, it is not known whether targeting to CBs is an absolute requirement for modification. To answer this question we analyzed modification of U1, U2, U4 and U5 snRNAs in Drosophila. We compared modification patterns in wild type flies, which have prominent CBs, to those in coilin-null flies, which lack detectable CBs. Despite the absence of CBs, snRNAs are properly modified in coilin-null flies. Northern blot analysis showed that the overall amount of snRNAs and of 7 box C/D scaRNAs, which direct 2'-O-methylation of snRNAs, is almost the same in wild type and mutants. In the mutants these RNAs are not concentrated in CBs, but are dispersed throughout the nucleus. In addition, we used Xenopus oocytes to develop two In Vitro assays for 2'-O-methylation. In one assay, In Vitro transcribed Drosophila snRNAs were mixed with their corresponding guide RNAs in an extract derived from germinal vesicles isolated in mineral oil. In the other, the two RNAs were injected into living oocytes. In both cases the snRNAs were properly methylated. It is well known that CB localization of scaRNAs requires a specific motif, the CAB box. We generated a mutated version of mgU2-28 scaRNA that lacks a CAB box. In the In Vitro systems mutant mgU2-28 RNA was fully functional as a guide RNA for 2-O-methylation of U2 snRNA. Our data demonstrate that neither the snRNAs nor their guide RNAs need to be concentrated in a cytologically detectable CB for efficient post-transcriptional modification to take place.

2392

Regulation of mRNA Stability and Translation by a Cell Fate Control System.

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The budding yeast Ndr/LATS family kinase Cbk1 is the downstream-most component of the RAM network, a conserved signaling system that controls daughter cell specific transcription and aspects of cell polarity. The kinase and its regulatory network are essential in cells that express wild type Ssd1, an RNase II-domain containing protein of unknown function implicated in numerous processes. We find that Cbk1 directly negatively regulates Ssd1 by phosphorylating consensus motifs in an N-terminal domain. Inhibition of Cbk1 results in lethal hyperactivation of Ssd1, causing a failure of cell growth due to inability to reorganize and expand the cell wall. No other processes involved in polarized growth are affected, including cytoskeleton organization or polarized secretion. Consistent with a role in mRNA metabolism, we find that Ssd1 is recruited to cytoplasmic P bodies. We have identified a specific subset of mRNAs that stably associate with Ssd1 in vivo, which largely encode proteins involved in morphogenesis and organization of the cell wall. The decay rate of a broad range of mRNAs is dramatically faster in cells that express Ssd1; Cbk1 inhibition increases Ssd1-dependent mRNA decay rate. However, this effect is not specific for messages with which Ssd1 associates. Rather, we find that mRNAs bound by Ssd1 are selectively depleted from polysome fractions when Cbk1 is inhibited, a treatment that does not affect global polysome profiles. Thus, Ssd1 likely acts as a translation repressor on bound mRNAs, and Cbk1 inhibits this function. Intriguingly, expression of several Ssd1-bound mRNAs is driven by the Cbk1-activated transcription factor Ace2, suggesting that the kinase functions in a feed-forward system by turning on transcription of mRNAs and directly inhibiting a protein that blocks their translation. Cbk1 also localizes to sites of cell growth and wall remodeling, indicating that this system may promote local stabilization and translation of Ssd1-associated mRNAs involved in these processes.

2393

The Role of Dicer's Helicase Domain.

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To gain a better understanding of the role of the helicase domain of C. elegans Dicer, we introduced a transgene encoding either wildtype Dicer (dcr-1), or dcr-1 with one of three different helicase mutations (K39A, D145N, G492R), into animals containing a homozygote deletion in the Dicer gene. Introduction of each construct rescued the sterile phenotype of these animals when grown at 20°C. All strains were able to mount a wildtype RNAi response to dsRNA introduced by feeding, and correspondingly, were able to produce exogenous small interfering RNA (siRNA). The finding that the helicase mutants were not deficient for exogenous RNAi led us to assay for defects in endogenous small RNA processing. We assayed piRNAs, 4 miRNAs, and 5 endogenous siRNAs (endo-siRNAs) by northern blot and saw no noticeable defects in processing either piRNAs or miRNAs. However, helicase mutant lines were devoid of four of the five endo-siRNAs tested. We also observed a corresponding increase in the miRNAs complementary to each of the four missing endo-siRNA. We next performed high throughput sequencing of small RNAs from wildtype and K39A rescue strains, using a protocol designed to look specifically at primary endo-siRNAs. This analysis indicated that the helicase domain is required for the accumulation of many, but not all, endo-siRNAs and confirmed that the helicase domain is not required for the processing of miRNA. Our model is that Dicer's helicase domain allows the enzyme to act processively, binding long dsRNA and cleaving along its length before release. A single double-stranded cleavage is sufficient to generate a miRNA from its short precursor dsRNA, explaining why the helicase domain is not required for miRNA processing. This model is based on In Vivo data, and we are now attempting to prove the model with In Vitro biochemical studies.

Minisymposium 31: Undergraduate Biology Curriculum in the 21st Century (2394 – 2399)

2394

A New, Interdisciplinary Foundation for the Life Sciences.
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In 2005-06, Harvard launched a new series of introductory courses in the life sciences, each developed by a team of faculty drawn from several departments across the biological and chemical sciences. The goal was to develop a new undergraduate curriculum that adequately addressed the many advances in the life sciences and the attendant interest in fostering interdisciplinary approaches to teaching and research. The courses therefore teach basic skills and knowledge, engage students in the most exciting aspects of the life sciences, and encourage them to think about the interconnectedness of the various scientific disciplines. We also sought to enhance retention in life sciences majors by offering an introductory experience that addresses important questions spanning multiple scientific fields today. The two courses, Life Sciences 1a and 1b, provide an interdisciplinary introduction to the Life Sciences for both majors and non-majors. LS 1a integrates fundamental topics in chemistry (general and some organic), molecular biology, and cell biology, while LS 1b covers basic genetics, genomics, probability and evolutionary biology. Each course includes an agreed upon inventory of basic concepts and skills developed from inter-departmental discussions that is then introduced in the context of exciting and interdisciplinary questions. Currently, the concepts of LS 1a are taught in the framework of understanding the possibility of synthetic life, and the biology and treatment of AIDS and cancer, while LS 1b focuses on different framing topics such as human population genetics and malaria. The combination of these life sciences courses, together with new interdisciplinary courses in the physical sciences, has replaced the traditional offerings in introductory biology and chemistry. Assessment of the educational outcomes of the new curriculum is ongoing, but preliminary data suggest that it has increased course enrollments, the number of life sciences majors, the motivation to take more advanced courses in science, and the ability to apply perspectives drawn from different fields to novel problems.
2395
Integrated Introductory Science Curriculum for Undergraduates at Princeton.
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For the past five years, we have offered a series of courses, taken in the freshman and sophomore years, that provide students with strong quantitative preparation for a major in any scientific discipline. The program is designed to help students retain the connections between their ultimate choice of major and the other disciplines. The curriculum is founded on the expectation that much of the most important science of the future, though based on the classical disciplines, will lie in areas that span two or more of them. The curriculum covers the core material of introductory physics, chemistry, biology (genetics and biochemistry), and computer science, all in an integrated manner in the equivalent of six semester courses taken in the first two years of college. The central role of mathematics as a universal language of science is emphasized throughout. Laboratory exercises introduce modern measurement techniques and illustrate major the quantitative principles they have mastered. The students study the dynamics of falling spheres in viscous fluids; they build, calibrate and use a simple photometer to measure bacterial growth; they measure mutation rates in yeast by the method of Luria and Delbrück, and determine directly Boltzmann's constant, by measurements of the Brownian motion of fluorescent latex spheres to a precision of the order of 5%. Primary data are generally obtained by digital image capture (directly or through a microscope). Students analyze their data using MatLab and Java programs, often of their own devising. The integrated science program is proving to be excellent preparation for a very broad range of majors (and ultimately, careers). About 1/2 the students major in some area of biology, about 1/3 in physics or computer science, with the remainder scattered over the remaining scientific disciplines and engineering. The curriculum is especially valuable for students interested in bridging the traditional barriers between the biological and the physical sciences. About half the students ultimately receive a Certificate in Quantitative Biology, which involves additional advanced courses, including a double-credit Project Laboratory in the MIT style.

2396
Mathematics Plus Biology Equals Improved Curriculum.
A. Campbell1,3, L. J. Heyer2,3, C. J. Paradise1; 1Biology, Davidson College, Davidson, NC, 2Mathematics, Davidson College, Davidson, NC, 3Genome Consortium for Active Teaching, Davidson, NC

Over the past decade, we have included more mathematics in our biology courses as well as more biology in our mathematics courses. We have blended our mathematics and biology research efforts in genomics, bioinformatics and more recently synthetic biology. Contrary to conventional wisdom, we have found that many students enjoy interdisciplinary biology and a more mathematical approach to learning biology. We have been successful at recruiting students of color into our research group on a campus that is 74% Caucasian and 87% of biology majors (1999 - 2009) have been Caucasian. Based on these experiences and a series of reports such as BIO2010, we developed a new approach to teaching introductory biology. All of biology can be encompassed by five big ideas: information, evolution, cells, emergent properties, and homeostasis. Biology also can be divided into five levels of organization: molecular, cellular, organismal, population, and ecological systems. Thus, we use a five by five matrix to present the key elements of biology while reducing excessive factoids. Research has shown that students remember more information if they: 1) construct their own knowledge, and 2) connect what they are learning to what they know. We use real data to let students construct their own knowledge. We use mathematics to illuminate biology and help students understand the value of mathematics. We incorporate ethical, legal and social implications to help students connect what they are learning to what they already know from every day life. In short, we are changing the way we teach biology.
New DNA sequences allow an examination of the details of genes and genome organization, critical parameters in the regulation of gene expression. The web-availability of enormous collections of sequences has created new opportunities for student-scientist research partnerships. Students can take ownership of stretches of sequence for improvement and annotation. These processes are labor-intensive, but with faculty guidance can provide an outstanding learning experience for undergraduate students, supported by the central resources of the Genomics Education Partnership (GEP). The GEP brings together faculty from over 50 colleges and universities in a collaborative project to provide their students with a research experience in genomics, making research a key part of a genetics/genomics lab course. The first project undertaken by the GEP is the sequence improvement and annotation of the dot chromosomes of several Drosophila species. This unique domain has properties of both euchromatin, including normal gene density, and heterochromatin, including a high density of repetitious DNA- remnants of transposable elements and retrotransposons. Undergraduates improve the posted sequence data to an error rate of less than 1:1000 bp, and generate carefully annotated gene models. The output from the students is assembled and used for analysis of the chromosome organization among different Drosophila species. Students appreciate the opportunity to make novel contributions to the scientific database that are likely to result in a scientific publication. A post-course survey given the past two years was highly reproducible, and confirmed that student personal gains were strikingly similar to those of students involved in a full-time summer research experience. Students also demonstrated learning gains on a quiz on genes and genomes. Positive self-assessment of learning correlated with student performance on the quiz. We find that genomics research is a rewarding way for us to teach, and a rewarding way for our students to learn. Funded by grants from HHMI and NIH.

A research based undergraduate laboratory curriculum builds upon and encourages the pursuit of scientific knowledge through contextualization. We present here a description of a complete redesign of the chemistry and biology laboratory program at Simmons College, a small liberal arts college for women in Boston. Based on the success of a pilot program integrating current faculty research into a sophomore organic chemistry course, we have introduced research-based laboratory integration across the curriculum. This new approach to laboratory training that we call Undergraduate Laboratory Renaissance, replaces the closed-ended laboratory experiments in selected chemistry, biology, and physics courses with faculty research projects that form the core for the semester’s laboratory work. All Simmons seniors are required to perform a yearlong senior research project. In the Life Sciences, this involves laboratory research under the guidance of a faculty member and culminates in a written thesis and oral defense before a faculty committee. The Laboratory Renaissance extends this research opportunity to students much earlier in their college careers and involves them in research throughout their college experience. Engaging students in scientific research earlier stimulates critical analysis, develops better laboratory skills, and provides a comprehensive “real-world” understanding of research. All leading to a great number of students going on to graduate studies in the life sciences and persisting in scientific careers.
2399

**StarBiochem: 3-D Protein Visualization in the Classroom.**
L. M. Aleman\textsuperscript{1,2}, C. Shubert\textsuperscript{2}, G. Walker\textsuperscript{1}; \textsuperscript{1}Biology, MIT, Cambridge, MA, \textsuperscript{2}Office of Educational Innovation and Technology, MIT, Cambridge, MA

Exploring protein structure/function relationships is an important teaching goal in core biology curricula. Classical teaching methods rely on two-dimensional static pictures or cartoon models to represent complex three-dimensional protein relationships, which may introduce misconceptions regarding protein structure/function relationships. One solution is to use available protein visualization tools to introduce students to three-dimensional structures. However, existing research-based visualization tools are not designed for educational use and require software installation and significant technical expertise. StarBiochem is a freely accessible (web.mit.edu/starBiochem), platform independent protein visualization tool that bridges the gap between current educational and research-based visualization tools. Unlike conventional viewers, the StarBiochem user interface visually represents protein structural information based on the four levels of protein structure. StarBiochem enables users to search and download molecules directly from the Protein Data Bank, including newly published structures. The StarBiochem website also provides ‘teaching modules’ based on a variety of protein structures (including aquaporin, DNA glycosylase, hemoglobin, GFP, and crystalline), enabling teachers to easily incorporate 3-d protein visualization in the classroom. StarBiochem teaching modules range in difficulty and are suitable for various educational settings. Current modules focus on protein structure, structure/function relationships and the connections between protein structure and disease. StarBiochem is currently used in introductory biology courses at MIT and Brandeis, high school outreach programs at the Broad Institute and the MIT Museum, the Boston Public School system, and teacher training programs in several states. We are currently concentrating on expanding usage of StarBiochem, providing educational support to teachers and faculty, and refining StarBiochem’s usability and capabilities. StarBiochem is part of a suite of research-based educational software (web.mit.edu/star), including StarGenetics (a virtual genetic lab), and StarORF (a six frame translator).

**Symposium 7 - Breaking the Diffraction Barrier (2400 – 2402)**

2400

**Nanoscopy with Focused Light.**
S. Hell; Department of NanoBiophotonics, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

For many decades, it has been accepted that diffraction of light precludes any lens-based optical microscope from discerning details smaller than half of the wavelength of light (~200 nm). However, in the 1990’s it was discovered that basic state transitions in a fluorophore can be exploited to eliminate the resolution-limiting role of diffraction. Since then, fluorescence microscopes have been developed that are able to resolve on the nanometer scale. Because they use focused visible light, these microscopes can image (live) cells noninvasively in 3D. Dubbed STED, SSIM, RESOLFT, PALM/STORM, etc., these concepts share a common basis: switching the ability of the dye to fluoresce on or off, such that inseparable features are registered sequentially in time. Although the various methods may utilize related or even identical fluorophore state transitions, they may substantially differ in the way the images are formed and the microscope is operated. Here, we discuss the basic principles of these ‘nanoscopy’ methods, along with their particular strengths and limitations. Likewise, we show initial applications in the life sciences. For example, STED microscopy has been employed to investigate the fate of synaptic vesicle proteins after exocytosis. A video-rate STED microscope was used to describe the mobility of vesicles inside the axons of cultured living neurons, evidencing that it is possible to visualize an intracellular process with nanoscale resolution in real-time. Live-cell STED microscopy has also been used to image activity-dependent morphological plasticity of dendritic spines, while in another study, it revealed that single sphingolipids, but not phospholipids, are
transiently (< 10 ms) and locally (< 20 nm) trapped in a living cell membrane. Altogether, lens-based optical nanoscopy is an arguably unexpected and fascinating development in the physical sciences that is poised to impact the life sciences, particularly cell biology, in the near future.

2401

High-speed Atomic Force Microscopy for Dynamic Visualization of Biomolecular Processes.

T. Ando; Department of Physics, Kanazawa University, Kanazawa, Japan

It has long been desired to directly watch biological macromolecules at work because such observation provides a straightforward way to understand how they function. Fluorescence microscopy can trace dynamic behavior of fluorescent spots emitted from individual labeled biomolecules but never allows us to observe biomolecules themselves. Atomic force microscopy (AFM) allows direct visualization of biomolecules in liquids at submolecular resolution but cannot image moving objects. To break this barrier, we have developed high-speed AFM (Ando et al., Proc. Natl. Acad. Sci. USA (2001) 98, 12468-12472). Submolecular resolution and high temporal resolution are attained simultaneously. Details of the new AFM technology are given in (Ando et al, Prog. Surf. Sci. (2008) 83, 337-437). We verified the reliability of this emerging technology by imaging dynamic structural changes in proteins for which many data had already been accumulated. This verification step is crucial for this new microscopy to be applied hereafter to a wide range of biological samples. for this verification, we studied a motor protein, myosin V (M5), and a light-driven proton pump, bacteriorhodopsin (bR). It is already established that M5 walks on actin filaments in a hand-over-hand manner; the two heads change their lead and trail positions alternately to walk. bR contains seven transmembrane α-helices (named A-G) enclosing retinal. Upon light absorption by retinal, the proton channel at the cytoplasmic surface is opened by the tilting of helix F away from the protein center, which is followed by rearrangement of the E-F interhelical loop. Bio-AFM visualization of these samples revealed molecular processes which basically agreed with previous results, verifying the reliability of the new microscopy. In addition, the captured images revealed more details of the processes unfindable with other approaches, giving deeper insights into the molecular mechanisms. Thus, directly visualizing livelily acting molecules by high-speed bio-AFM was demonstrated to be a powerful new approach to study elaborate biomolecular processes.

2402

Super-Resolution Imaging with STORM.

X. Zhuang; Department of Chemistry and Chemical Biology, Harvard University/HHMI, Cambridge, MA

When combined with a large repertoire of fluorescent probes, light microscopy allows the direct visualization of molecular processes in living organisms. The diffraction-limited resolution of fluorescence microscopy, however, leaves many biological structures too small to be observed in detail. Here, I describe a new imaging method, stochastic optical reconstruction microscopy (STORM), which breaks the diffraction limit and allows for super-resolution imaging. STORM uses single-molecule imaging and photo-switchable fluorescent probes to temporally separate the spatially overlapping images of individual molecules, thereby breaking the diffraction limit. Using this approach, we have imaged cellular structures with a few tens of nanometer resolution in 3D. In this talk, I will discuss the general concept, recent technical advances, and various biological applications of STORM.