Chicago Cytoskeleton

Friday March 15, 2024 3:00-7:00 pm

Four talks *and* the yearly poster session/competition!

- **Noah Mitchell** (KITP; soon at University of Chicago)
  How biology uses physics to sculpt an inner organ
- **Katie Drerup** (University of Wisconsin - Madison)
  HOPS-mediated vesicle fusion is essential for initiation of autophagosome transport
- **Dileep Varma** (Northwestern Feinberg School of Medicine)
  The Hexosamine Biosynthetic Pathway is a key regulator of cell proliferation and migration in metastatic prostate cancer
- **Greg Smith** (Northwestern Feinberg School of Medicine)
  Regulation of the kinesin-1 motor by alphaherpesviruses

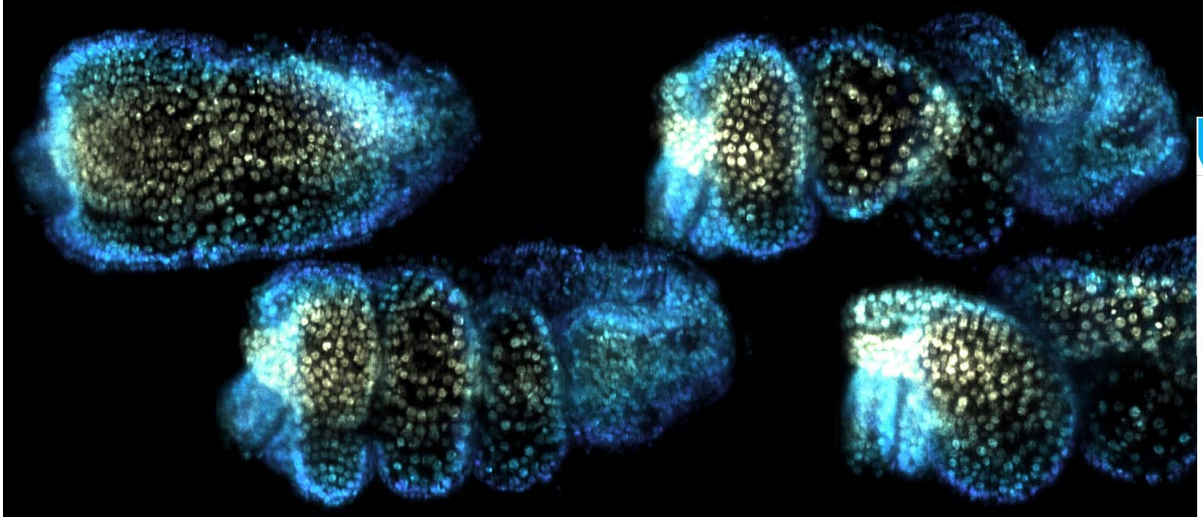

Image: Noah Mitchell & Streichan Lab

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303 E. Superior
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Tricellular Zipping: A novel process in epithelial cell-cell junction remodeling

Babli Adhikary and Ann L. Miller
Department of Molecular, Cellular, and Developmental Biology, University of Michigan,
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Tissue morphogenesis, homeostasis, and disease progression are often accompanied by loss and/or gain of cell-cell contacts between neighbor cells within an epithelial sheet. Notable examples of such topological changes include cell intercalation and rosette resolution during embryogenesis. In *Xenopus laevis* embryos, the transition to gastrulation entails a transformation of epithelial cells from irregular extended shapes to a more ordered hexagonal packing arrangement. During this transition, we recently observed a novel process, which we named **"tricellular zipping"**, whereby epithelial cell extensions at the sites of higher-order vertices (points of convergence of four or more cell vertices) are resolved as two cells zip together via lengthening their bicellular interface, and the other cell recedes. In our preliminary investigations, we have identified **calcium** as a novel molecular participant in the zipping process. Multiple calcium flashes occur in the receding cell over the course of several minutes. Additionally, we have observed an increase in the local intensity of F-actin in the receding cell, which we speculate may be responsible for pulling the two junctions together. Finally, we have detected an accumulation of the tight junction protein ZO-1 and the tricellular tight junction protein Tricellulin, both moving in the direction of the zipping process, alongside an enrichment of active Rho GTPase in the zipping cells. Our ongoing research is aimed at understanding how actin contractility, driven by calcium signaling, facilitates the successful remodeling of higher-order junctions within epithelial tissues. We propose that tricellular zipping may be needed to maintain adhesion and barrier function during post-cytokinesis junction reorganization. Understanding the underlying mechanisms and the functional significance of tricellular zipping will help us gain a better understanding of the regulation of junction remodeling in epithelial tissue as cells undergo dynamic changes in their packing order.

Name: Kashmeera D Baboolall

Biophysical Sciences PhD Candidate

Kovar and Gardel Labs

University of Chicago

Sensing the force: Building an in-vitro reconstitution assay to probe the force-dependent response of mechanosensitive actin-associated proteins

Mechanical forces function as cellular inputs and outputs that enable the proper execution of essential biological processes. The actin cytoskeleton is a mechanochemical conduit that senses and transmits mechanical cues throughout the cell, which responds by triggering biochemical changes. The actin cytoskeleton thus plays an important role in mechanotransduction. While the importance of mechanotransduction pathways is well-established, the underlying molecular mechanisms behind mechanosensing are still poorly understood. Recently, the mechanosensitive behavior of some LIM (Lin-11, Isl-1, MEC-3) domain-containing proteins has been reported due to these proteins' enriched localization to stressed actin filaments. Studying the molecular underpinnings of this novel interaction is key to our understanding of mechanosensing. The establishment of an in-vitro reconstitution assay that clearly captures and characterizes the force-dependent localization of LIM domain proteins on single actin filaments is thus essential to gain insight into the molecular mechanism of force-activated binding of proteins to actin filaments. In this study, we tested the ability of several synthetic myosin motors to generate mechanical stresses on single actin filaments for the recruitment of mechanosensitive LIM domain proteins. We used TIRF (Total Internal Reflection Fluorescence) microscopy paired with a single molecule particle analysis pipeline to visualize and identify the localization of some LIM domain proteins to stressed actin filaments. Since synthetic myosin motors can be engineered to have modular characteristics, this in-vitro reconstitution force assay provides some level of control over the stresses exerted on individual actin filaments. The current in-vitro reconstitution force assay is thus promising for the understanding of the molecular mechanism involved in the sensing of stressed actin filaments by mechanosensitive proteins.

ABSTRACT

Correlating ciliogenesis rate with kinesin-2 (KIF3A/KIF3B) expression level

Shivani Batra, Lindsey Margewich, Jessica Adams, and Martin F. Engelke

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Kinesin-2 is a plus end-directed motor protein comprising the motor domain containing subunits KIF3A and KIF3B and the accessory protein KAP3. It is crucial for intraflagellar transport (IFT), ciliogenesis, and cilia maintenance. Several studies report differential gene expression of KIF3A and/or KIF3B in tumor tissue. Furthermore, in a context-specific manner, the gain and loss of ciliogenesis have been suggested as a key step toward malignancy in several cancer types. If or how the kinesin-2 expression level affects ciliogenesis is currently not known. Here, we propose to use a well-characterized *Kif3a*$^{-/-}$ *Kif3b*$^{-/-}$ double knockout mouse embryonic fibroblast cell line to address this question. To measure motor expression on a single cell level, we will express KIF3A and KIF3B N-terminally tagged with the extremely bright fluorescent proteins tdStayGold and tdTomato, respectively. The fluorescence intensity in the green and red channels linearly correlates with KIF3A and KIF3B expression levels. We will serum-starve the cells for two days to stimulate ciliogenesis, fix the cells, and label cilia by immunofluorescence against the ciliary membrane marker ARL13B and the axoneme marker acetylated tubulin. Next, hundreds of fluorescence micrographs will be automatically analyzed using a machine-learning approach developed in collaboration with the AI4Life consortium, allowing us to correlate motor expression level with ciliogenesis rate. These data have the potential to inform the diagnostics and treatment of patients.

T cells Use Focal Adhesions to Pull Themselves Through Confined Environments

Alexia Caillier , David Oleksyn , Deborah J Fowell , Jim Miller , Patrick W Oakes

Immune cells are highly dynamic and able to migrate through environments with diverse biochemical and mechanical composition. Their migration has classically been defined as amoeboid under the assumption that it is integrin-independent. Here we show that activated primary Th1 T cells require both confinement and extracellular matrix protein to migrate efficiently. This migration is mediated through small and dynamic focal adhesions that are composed of the same proteins associated with canonical mesenchymal focal adhesions, such as integrins, talin, and vinculin. These focal adhesions, furthermore, localize to sites of contractile traction stresses, enabling T cells to pull themselves through confined spaces. Finally, we show that Th1 T cell preferentially follows tracks of other T cells, suggesting that these adhesions are modifying the extracellular matrix to provide additional environmental guidance cues. These results demonstrate not only that the boundaries between amoeboid and mesenchymal migration modes are ambiguous, but that integrin-mediated adhesions play a key role in T cell motility.

Deciphering the role of RhoA and cell geometry in septin recruitment to the actin cortex

- Shreya Chandrasekar, Jordan R. Beach, Patrick W. Oakes

The cytoskeleton provides an architectural framework which cells use to generate and respond to physical forces. Historically, actin, microtubules and intermediate filaments have been the most well studied components of the cytoskeleton. More recently, septins have been recognized as a fourth important component of the cytoskeleton. These GTP-binding proteins not only localize to a subset of actin stress fibers, but also to regions that are thought to need mechanical reinforcement (e.g., base of cilia and blebs). In many of these regions there is also a concomitant enrichment of active RhoA (e.g. the cleavage furrow). This suggests septins could be playing a role in either altering or sensing local mechanical properties of the cytoskeleton. While the preferential localization of septins to micron-scale membrane curvatures has been previously shown, the mechanism by which septins are recruited to these structures is yet to be considered. Here we test whether septins are recruited in response to RhoA activation, changes in cell geometry and mechanics, or a combination of the these signaling pathways. To test the effect of local RhoA activation on septin relocalization, we use an optogenetic probe and show an enrichment of septin filaments in regions of local RhoA activation in adherent cells. Conversely in non-adherent cells with locally active RhoA, we do not see septin colocalization at regions of active RhoA. Interestingly, when cells migrate through microchannels featuring constrictions, septins accumulate along the cortex at the site of constriction. This suggests that septins relocate to sites of locally active RhoA in adherent cells and along sites of cortical compression. Together these data help to reveal the signaling mechanisms that regulate septin localization in the cytoskeleton.

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Title: Anillin Regulates RhoA Activity during Cell-Cell Junction Remodeling

Authors: Zie Craig, Kelsey Walworth, Alexander Walkon, Rachel Stephenson, Torey Arnold, Ann L. Miller

Affiliations: University of Michigan

Epithelial tissues require apical cell-cell junctions to adhere cells to one another and generate specialized barriers that are essential for homeostasis, while disrupted barrier function is linked to disease pathogenesis. The mechanisms underlying junction maintenance, repair, and remodeling are not fully understood. Using Xenopus embryos as a model epithelium, our lab found that tight junctions rapidly repair following local leaks in the barrier through a process we termed Rho flares. Rho flares are short-lived, local accumulations of active RhoA which trigger actomyosin contractility to restore barrier function. Anillin is a scaffolding protein known to regulate RhoA during cytokinesis, where it forms a tension-dependent complex with p190RhoGAP (p190) to terminate Rho contractility after cytokinetic furrow ingression is complete. We and others have shown that Anillin also localizes to apical cell-cell junctions, where it regulates junction integrity, organizes junctional actomyosin, and perpetuates RhoA activity by increasing RhoA's membrane retention time and thus its availability to downstream effectors. Here, we show that both Anillin and p190 localize to RhoA flares. Therefore, we hypothesize that during Rho flares, Anillin retains active RhoA at the plasma membrane so that sufficient contractility can been reached, and recruits p190 to limit excess Rho-mediated contractility. We found that Anillin knockdown increases the intensity of Rho flares, which suggests that Anillin is normally involved in negative regulation of RhoA. However, Anillin knockdown also reduces the duration of Rho flares, and tight junctions are not reinforced following tight junction breaks. Importantly, these changes in Rho flare dynamics increase the frequency and size of barrier leaks. Ongoing work is focused on determining the mechanism underlying Anillin and p190's recruitment and function at Rho flares. We are investigating: 1) the requirement of Anillin binding to RhoA for Anillin's localization to Rho flares and whether this binding is required for successful junction repair, 2) the function of p190 at Rho flares, and 3) whether p190's interaction with Anillin is required for p190's recruitment and localization pattern at Rho flares. Together, this work will reveal how Anillin and p190 coordinate contractility during RhoA-dependent tight junction repair.

Ndel1 modulates dynein activity in multiple ways

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Cytoplasmic-dynein-1 (dynein) is a microtubule minus-end directed motor protein that facilitates most microtubule-based trafficking and promotes spindle alignment and focusing. To become active, dynein binds to the dynactin complex and an adaptor to form the “activated dynein complex”; the protein Lis1 promotes formation of the activated dynein complex. Ndel1 is a dynein- and Lis1-binding protein that has been shown to help coordinate dynein localization in the cell. Cell-based assays also suggest that Ndel1 may work with Lis1 to facilitate dynein activation; however, Ndel1 has been shown to compete with dynactin for dynein binding, suggesting an inhibitory role for Ndel1. In this study, we examined the mechanisms of Ndel1 in dynein regulation using single molecule TIRF microscopy and protein biochemistry. We determined that Ndel1 hinders formation of the activated dynein complex and observed that Ndel1 interacts with dynein and Lis1 in a way that prevents Lis1-mediated dynein activation. Together, our work suggests that Ndel1 is a negative regulator of dynein activation in vitro. In future studies, we will use negative stain- and cryo-electron microscopy to gain further insight into the binding interactions of Ndel1 with dynein.

Role of WASP in Arp2/3 complex activation

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Actin filament branching is a fundamental cellular mechanism generating forces necessary for lamellipodia and filopodia formation critical for cell migration and motility. Filament branching is nucleated on the sides of preexisting actin filaments by Actin-Related Protein 2/3 (Arp2/3). Arp2/3 protein, natively found in inactive state, is activated by nucleation promoting factors (NPFs). Arp2/3 adopts a barbed end like conformation and binds to the actin mother filament to function as a nucleation seed for daughter filament growth [1,2]. The role of NPFs and actin filaments in the activation of Arp2/3 and the conformational rearrangements occurring during Arp2/3 activation remains highly debated. In this work, we present a molecular dynamics study investigating Arp2/3-NPF interactions at molecular scales. By sampling inactive-active state transition using well-tempered metadynamics simulations we find that the energy barrier impeding short-pitch conformational transition of Arp2-Arp3 results from the interaction of subdomain 2 of Arp2 with the "bumper helix" in Arp3. Further, using a coarse grained heteroelastic network model derived to reflect the correlated fluctuations observed at atomic resolutions [3], we aim to capture the slow "soft" modes of the multidomain protein resulting in the activation of Arp2/3 complex. Overall, using atomistic and coarse grained simulations, our study provides molecular insights to unravel the mechanism of Arp2/3 activation.

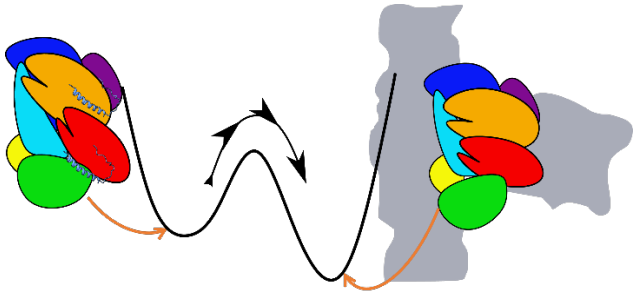

Figure 1: Schematic of mechanism of Arp2/3 activation.

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Abstract Text:
Gastrulation is a pivotal developmental stage in humans, marking the emergence of bilateral symmetry and the transformation of the blastula into a complex, multilayered embryo with three germ layers. Nestled between ectoderm and endoderm, the mesoderm plays a central role forming a diverse variety of critical cell types. Mesodermal cells orchestrate collective cell migration in vivo, relying on dynamic cytoskeletal reorganization to respond to mechanochemical cues during their journey from the primitive streak. However, the fragility of in vivo embryos, limited non-lethal genetic manipulations, and inherent cellular complexities have posed significant challenges in studying this dynamic process. To this end, we leverage bioengineering techniques, specifically micropatterning and fabrication, to control and standardize tissue size and shape. Using gastruloids derived from human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) to reconstruct key gastrulation events, we gain precise control over biomechanical cues, unveiling temporal and spatial kinetics. This comprehensive approach underscores the intricate interplay of tissue-wide tension, cytoskeletal dynamics, and adhesion-based signaling in shaping mesoderm behavior and fate.

The roles of Arp2/3 complex nucleation promoting factors in diverse actin cytoskeleton networks in the early C. elegans embryo

Liam Leeming[1], Sarah Yde[2], Rachel Kadzik[2], Ed Munro[2,3,4], David R. Kovar[2,3,4,5]

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Many different actin binding proteins (ABPs) are involved in regulating the assembly of actin filaments (F-actin) into a wide array of structurally and functionally distinct networks. Arp2/3 complex assembles branched F-actin networks that typically provide pushing forces in contexts such as cell motility or endocytosis. In the single-cell *C. elegans* embryo, Arp2/3 complex concurrently assembles branched filaments in multiple networks, such as endocytic “mini-comets” and the base of extending filopodia. Thus, Arp2/3 complex needs to be activated at different times and places within the cell by VCA domain containing nucleation promoting factors (NPFs). In *C. elegans* WAVE complex and WASP (*wsp-1*) are the two best studied NPFs, but it is still not well understood how Arp2/3 complex is differentially activated by WAVE and WASP to facilitate the assembly of different branched actin networks, especially in the context of a single cell.

Using two-color TIRF microscopy, we visualized endogenously tagged fluorescent versions of each NPF, and filamentous actin using fluorescently labeled LifeAct. We observed that both WAVE and WASP are both enriched at the anterior cortex, but with distinct localization patterns. WASP localizes to the leading edge of mini-comets as well as to the base of filopodia. Interestingly, WAVE is enriched at the tips of filopodia, and the leading edge of a subset of actin comets that are observed to have different dynamics than those associated only with WASP. RNAi knockdown of WAVE complex subunit wve-1 significantly decreases the number of both filopodia and WAVE rich comets.

These results suggest that there are two distinct populations of mini-comets in the *C. elegans* single cell embryo, and that there may be interactions between WAVE complex and the formin cyk-1, which has also been observed at the tips of filopodia. These data also suggest that filopodia may contain multiple branched F-actin networks, nucleated by different NPFs and playing distinct roles in regulating filopodial assembly, maintenance, and disassembly. However, because we do not observe Arp2/3 complex at the tips of filopodia, it is also plausible that WAVE complex has an Arp2/3 complex independent role in filopodia.

Probing the mechanism of dynein activation

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Dynein is the primary retrograde directed microtubule-associated motor in most eukaryotes. Unlike many motor proteins, dynein is not active until it is bound to additional regulatory factors. To achieve activation, dynein must bind to the protein complex dynactin and one of a family of adaptors to form a transport complex capable of binding cargo and moving processively along the microtubule. Although much is known about the components that are required for dynein activation, how this complex assembles remains unresolved. To uncover mechanistic details about the steps that precede dynein activation, we developed a single-molecule fluorescence microscopy assay that allows for visualization of dynein-dynactin-adaptor binding in real-time. Using this assay, we have measured the kinetics of complex formation with two different adaptors and determined the order of component assembly required for efficient activation. Our preliminary data suggests that the order of addition of individual components dictates the duration that both dynactin and adaptor remain bound to dynein. Together, these studies provide insight into the sequence of molecular events that facilitate dynein activation and have far reaching implications for how dynein activation may be regulated in a cellular environment.

Chicago Cytoskeleton Abstract March 2024

Maddie Lovejoy, Agustin Rabino, Vennela Gangasani, Sophia Durham, and Rafael Garcia-Mata

University of Toledo

Most internal organs consist of a polarized epithelium surrounding a central lumen, which separates the interior of cells from the external environment. The establishment of polarity is essential for epithelial cells' function, and abnormalities in this process are a hallmark of many diseases, such as cancer. One of the critical regulators for cell adhesion and polarity is the Scribble complex, which is a protein complex comprised of Scribble, Dlg1, and Lgl.

We previously showed that Scribble and Dlg1 can form a ternary complex with the RhoG-specific GEF, SGEF, which also functions as a scaffolding protein. In epithelial MDCK cells cultured in 2D, we showed that SGEF plays a role in E-cadherin transcription, E-cadherin stability at adherens junctions, and tight junction integrity. Here we explore the role of SGEF during lumen formation in 3D cysts. Single MDCK cells embedded in Matrigel develop into multicellular, polarized 3D cysts over time, and are used as a model to study polarity. We found that mature SGEF knockdown cysts regulate E-cadherin levels through increased expression of Slug, which is an important E-cadherin transcriptional repressor during metastasis. These cysts also develop multiple closed lumens partially due to improper orientation of cell division. In addition to possessing abnormal cyst morphology, observing SGEF KD cyst development with live 4D spinning disc microscopy displayed an E-cadherin-mediated increase in collective motility and cyst fusion. Despite having pronounced contractile stress fibers, SGEF KD cells also migrated faster and, in preliminary experiments, showed a higher degree of intercalation in a 2D wound healing assay. We found that these atypical motility and lumen phenotypes can be partially or completely rescued when Slug is knocked down in SGEF KD cysts, which restores E-cadherin levels.

Our results suggest that, because Slug expression is increased in SGEF KD cysts, the consequential compromise of adherens and tight junctions is a major contributing factor to abnormalities in the morphology and motility of the cysts. A decrease in cell-cell adhesion has also been observed in most cases of metastasis, suggesting that SGEF could play a role in maintaining epithelial stability in MDCK cells. We plan to continue to explore the development and motility of cysts in the absence of SGEF by utilizing cells that fluorescently express apical, tight junction, and nuclear markers in future experiments.

"Mitotic" kinesin-5 is a dynamic brake for axonal growth

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Abstract

During neuronal development, neurons undergo dramatic microtubule reorganization to shape axons and dendrites intricately, establishing the structural foundation for efficient signal transduction in the nervous system. Previous studies from our laboratory have highlighted the unique role of kinesin-1 (conventional kinesin) in driving microtubule-microtubule sliding, supplying essential mechanical forces for initial outgrowth and axonal regeneration in *Drosophila melanogaster*. In this study, we unveil the critical involvement of kinesin-5, a mitotic motor, in the proper development of postmitotic neurons.

Kinesin-5 is a conserved homotetrameric motor that slides antiparallel microtubules apart in the bipolar spindle during mitosis. Here, we show that the *Drosophila* kinesin-5 homolog, Klp61F, is expressed in the larval brain neurons, mostly abundantly in the ventral nerve cord (VNC) neurons. Knockdown of Klp61F by a pan-neuronal driver results in severe locomotion defects and complete lethality in adult flies, primarily attributed to the absence of kinesin-5 in VNC motor neurons during early larval stages. Klp61F depletion causes major axon growth defects both in culture and *in vivo*. The adult lethality and axon growth defects can be fully rescued with a chimeric human-*Drosophila* kinesin-5 motor that accumulates at the axon tip. Furthermore, our study reveals an increased penetration of microtubules into the actin-rich lamellipodia region in Klp61F-depleted cells.

Altogether, our findings propose a multistep mechanism for kinesin-5 to reach the axonal growth cone. At the growth cone, kinesin-5 acts as a brake for kinesin-1-driven microtubule sliding, preventing premature microtubules from entering the periphery zone. This regulatory role of kinesin-5 is crucial for precise axon pathfinding during nervous system development, providing novel insights into the orchestration of microtubule organization in neuronal morphogenesis.

Title: The ALS-associated NEK1 kinase regulates microtubule stability and axonal transport in human iPSC-derived motor neurons

Authors: **Jacob R. Mann[1]**, Elizabeth D. McKenna[1], Darilang Mawrie[2], Vasileios Papakis[1], Francesco Alessandrini[1], Eric N. Anderson[2], Ryan Mayers[1], Hannah E. Ball[1], Evan Kaspi[1], Katherine Lubinski[1], Desiree M. Baron[3], Liana Tellez[1], John E. Landers[3], Udai B. Pandey[2], Evangelos Kiskinis[1,4,5]

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the loss of motor neurons (MNs) in the brain and spinal cord. Breakthrough genetic studies of familial ALS patients have enabled the identification of causative mutations in genes that may play a role in disease pathogenesis. Recently, loss-of-function variants in a novel identified gene *NEK1*, which encodes the NIMA-related kinase 1 (NEK1) protein, have been demonstrated to confer susceptibility for up to 3% of all ALS patients and can thus be considered a major genetic cause of ALS. While little is known about the role of NEK1 in normal MN physiology, preliminary evidence from our lab has implicated a dysregulation of microtubule (MT) homeostasis in NEK1 loss-of-function iPSC-derived MN models.

Using unbiased mass spectrometry-based proteomics analyses, we discovered that NEK1 interactors and protein expression changes in response to NEK1 loss-of-function converge on proteins enriched for function in the microtubule (MT) cytoskeleton. We have additionally identified alpha-tubulin (TUBA1B), a major structural component of MTs, as a NEK1-interacting protein that undergoes phosphorylation by NEK1 *in vitro*. Follow-up unbiased phosphoproteomic analysis in conditional NEK1-knockout cells revealed a reduction in phosphorylation of TUBA1B at the T56 residue, which we then generated an antibody against and confirmed diminished phosphorylation in NEK1 loss-of-function iPSC-derived MN models. We then performed a series of functional assays to assess the effects of NEK1 loss-of-function on the MT network and discovered a reduction in MT stability associated with NEK1 reduction (via siRNA) or ALS-linked NEK1 mutations in iPSC-derived MNs. This phenotype is accompanied by decreases in MT detyrosination and acetylation, two post-translational tubulin modifications associated with long-lived and/or stable MTs. NEK1 loss-of-function also resulted in compromised neurite regeneration following axotomy and alterations in axonal transport in iPSC-derived MNs. Together, these data suggest that NEK1 may function in the regulation of MTs in human MNs, and that ALS-associated *NEK1* mutations may contribute to degeneration of MNs through disruptions in MT stability.

Characterizing the Mechanism of Primary Cilia Disassembly Following Kinesin-2 Inhibition

Lindsey Margewich and Martin F. Engelke

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Primary cilia are hair-like protrusions on the surface of cells that play a role in cell signaling, motility, and development. Understanding primary cilia assembly and disassembly is important because malfunctions in these processes are linked to a group of human diseases called ciliopathies, with symptoms including obesity, cystic kidney disease, and abnormal development. Kinesin-2, consisting of KIF3A and KIF3B, is a heterodimeric motor involved in cellular transport into cilia along microtubules, also called intraflagellar transport. A previous study determined that upon inhibition of kinesin-2, intraflagellar transport stops, and cilia disassemble (Engelke et al., 2019). However, the mechanism of cilia disassembly is currently unknown. We aim to understand the mechanism of primary cilia disassembly when kinesin-2 inhibition blocks intraflagellar transport. To do this, we generated a 3T3 cell line stably expressing the inhibitable kinesin-2 along with the ciliary membrane marker SSTR3-mCherry. We visualize axonemal microtubules via SPY650-tubulin. Via time-lapse imaging, we observed that cilia disassembled by different mechanisms including ciliary shrinking and whole cilium shedding. We are currently growing this live imaging dataset to quantify the different disassembly mechanisms. To further dissect the ciliary disassembly mechanism, we will test if inhibition of candidate proteins or processes will slow or abolish cilium loss following kinesin-2 inhibition. Understanding how ciliary disassembly can be induced and what mechanism leads to cilium loss might inform future therapies for ciliopathies.

Title: The role of tension sensing in the development of the *Xenopus laevis* embryonic ciliated epithelium

Authors: Camilla Mastela[1], Lenore Pitstick[2], and Rosa Ventrella[3]

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Abstract: In recent years, our collective understanding of how smoking and pollutants cause lung and respiratory problems has continued to grow. However, lung tissue development and maintenance is not fully understood and is essential in delineating the negative effects of these harmful substances. The ciliated epithelium, located on the skin of *Xenopus laevis* embryos, has characteristics like human lung epithelium. One vital component in the ciliated epithelium is studying how cells respond when they experience tension from their surroundings throughout development. Due to the importance of mechanotransduction, we predicted that there are several tension-sensing pathways involved in the development of the *Xenopus laevis* ciliated epithelium, such as the Piezo1, Rho/ROCK, and Hippo/Yap. Certain activators and inhibitors of these pathways were used to determine their effect on ciliated epithelial development. Here we show that an inhibitor of Piezo1, GSMTX (5 μM), decreased the number of multiciliated cells (MCCs) present in the epithelium, but did not affect the apical area of the cells themselves. Activation of Piezo1 with the use of Yoda1 (100 μM) and inhibition of ROCK with the use of y-27632 (100 μM) did not affect the development of the ciliated epithelium. Also, we see that the downstream effector of the Hippo pathway, Yap, is localized in the nucleus of the outer cells, but the cytoplasm of MCCs, possibly suggesting that this pathway may play a role in the development of the ciliated epithelium. We anticipate that a better understanding of how these mechanotransduction signaling pathways contribute to the formation of the ciliated epithelium will give insight into how these pathways may be perturbed in the development of lung diseases.

Balancing competing effects of tissue growth and cytoskeletal regulation during *Drosophila* wing disc development

Mayesha Sahir Mim[1], Nilay Kumar[1], Jennifer Rangel Ambriz[2,3], Kevin Tsai[2,3], Marycruz Flores Flores[1], Weitao Chen[2,3], Jeremiah J. Zartman[1,4,*], Mark Alber[2,3,*]

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Abstract

How a developing organ robustly coordinates the cellular mechanics and growth to reach a final size and shape remains poorly understood. Through iterations between experiments and new model simulations that include a mechanistic description of interkinetic nuclear migration, we show that the local curvature, height, and nuclear positioning of cells in the *Drosophila* wing imaginal disc are defined by the concurrent patterning of actomyosin contractility, cell-ECM adhesion, ECM stiffness, and interfacial membrane tension. We show that increasing cell proliferation via different growth-promoting pathways results in two distinct phenotypes. Triggering proliferation through insulin signaling increases basal curvature, but an increase in growth through Dpp signaling and Myc causes tissue flattening. These distinct phenotypic outcomes arise from differences in how each growth pathway regulates the cellular cytoskeleton, including contractility and cell-ECM adhesion. The coupled regulation of proliferation and cytoskeletal regulators is a general strategy to meet the multiple context-dependent criteria defining tissue morphogenesis.

Determining how physiological profilin-actin concentrations and PIP2 affect G-actin partitioning between Arp2/3 complex- and formin-mediated F-actin network assembly

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Actin-binding proteins (ABPs) regulate filamentous actin (F-actin) elongation, architecture, and stability, which facilitate the formation of distinct F-actin networks for diverse fundamental cellular processes. Formin and Arp2/3 complex compete for a limiting globular actin (G-actin) pool, which temporally controls F-actin network assembly by incorporating G-actin into different F-actin assemblies. The small ABP profilin regulates G-actin competition by favoring G-actin partitioning into formin- over Arp2/3 complex-mediated networks. Profilin binds most of the G-actin pool to maintain an unpolymerized G-actin population and partitions G-actin in fission yeast to accelerate formin elongation but inhibits Arp2/3 complex branching; however, profilin's Arp2/3 complex inhibition mechanism is of debate. I will use in vitro spontaneous actin assembly assays with fission yeast formins, Arp2/3 complex, and profilin-actin visualized with Total Internal Reflection Fluorescence Microscopy (TIRFM) to determine how profilin affects competition between Arp2/3 complex and formin for G-actin as well as how physiological profilin-actin concentrations affect formin elongation and Arp2/3 complex branching. Additionally, phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$ or PIP2) has been proposed as a profilin sequesterer that may favor Arp2/3 complex- over formin-mediated assembly by freeing G-actin for use by the Arp2/3 complex near the membrane; however, PIP2's role in G-actin partitioning has not been studied. I plan to complete in vitro biochemical assays with lipid-coated microwells visualized with TIRFM to determine whether PIP2 is sufficient to influence G-actin partitioning. Understanding profilin's Arp2/3 complex inhibitory mechanism and whether PIP2 affects G-actin partitioning is critical to understanding how profilin and PIP2 can exert spatial-temporal control of F-actin network assembly.

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Title: Delineating the Intracellular Activity Profile of the Heterodimeric Kinesin-2

The heterodimeric kinesin-2 motor (KIF3A/KIF3B) has essential roles in many intracellular activities and is a critical component of intraflagellar transport (IFT). This transport along the axonemal microtubules of flagella and cilia is essential for their formation, function, and maintenance. While it is known that kinesin-2 motor activity is regulated by autoinhibition, how and where this motor is switched on and off has yet to be understood. Preliminary data from our lab confirm that kinesin-2 is autoinhibited by a mechanism similar to that of other kinesins. A hinge in the rigid coiled-coil stalk domain of the motor molecules facilitates molecular back folding, facilitating the inhibitory interaction of the tail portion of the motor with the motor domains. Unknown cellular cues relieve this inhibitory interaction, allowing the motor to assume the extended, active conformation in which the tail binds to cargo, while the motor domain mediates cargo transport by 'walking' along microtubules. We hypothesize that autoinhibited kinesin-2 diffuses to the base of the cilium, where it is activated for IFT. To test this hypothesis, we develop a sensor kinesin-2 that drives IFT similarly to the wildtype motor but changes its fluorescence profile depending on its activity status. For this, we fused parts of fluorescent proteins capable of bimolecular fluorescence complementation (BiFC) as sensor domains to the N- and C- terminus of KIF3B. While the fusion of the sensor domains to wildtype kinesin-2 results in BiFC, fusion of those domains to a hinge-deleted kinesin-2 does not. These results demonstrate that the sensor kinesin-2 faithfully reports motor activity. High-resolution imaging will allow us to construct a cellular activity map of kinesin-2 and reveal where this motor is activated for IFT.

Developing localization-based biosensors for active Rac1 and RhoG

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The Rho family of small GTPases control a wide range of cellular processes, including motility, polarity, axon guidance, and cell division. Rho GTPases cycle between an inactive GDP-bound and an active GTP-bound form. The activation of Rho proteins is catalyzed by RhoGEFs (guanine nucleotide exchange factors), whereas RhoGAPs (GTPase activating proteins) mediate their inactivation. Since the dynamics of Rho GTPase activation occur on a temporal scale of minutes and a spatial scale of micrometers, it requires tools that can measure their dynamic at these specific scales. Steady state readouts, such as the Rho affinity pulldown assay, cannot detect subtle patterns of spatiotemporal regulation. Similarly, complex spatiotemporal Rho GTPases networks, which include extensive crosstalk between Rho GTPases, require tools that can be multiplexed to visualize multiple components and/or pathways simultaneously. To address these issues, researchers have engineered genetically encoded fluorescent biosensors that allow the visualization of the activation state of Rho GTPases in single living cells with high spatiotemporal resolution. There are two types of sensors currently in use to analyze the spatiotemporal dynamics of Rho GTPases: Förster Resonance Energy Transfer (FRET)-based sensors, and effector localization sensors. Both types of sensors use a GTPase binding domain (GBD), which has a higher affinity for the active state of its cognate Rho GTPase. Localization-based biosensors used a fluorescent protein fused to a GBD specific for a particular Rho GTPase. One of the advantages of using localization-based biosensors is their simple design, as they only need a single fluorescent protein, allowing the combination of different biosensors and/or the use of a biosensor combined with other techniques for example, optogenetics. Another important advantage of using these biosensors is the ability to visualize Rho activity at endogenous levels, without altering their location in the cell. Here, we designed localization-based biosensors for active Rac1 and RhoG, for which there are no localization sensors available. By doing live imaging experiments, we systematically characterized the efficiency of these biosensors tagged with different fluorophores and validated their specificity in comparison with others Rho GTPases. In parallel, we validated their specificity biochemically using GST-pull down assays with constitutively active Rho GTPases. Once validated, these tools will allow us to characterize the regulation of the activity of endogenous Rac1 and RhoG in live cells in different cellular processes, including cell migration, adhesion and invasion.

Single-cell mechanical analysis reveals viscoelastic similarities between astrocytes and glioblastoma cells

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Despite recent advances in treatment, the survival rate of glioblastoma (GBM) patients remains low due to the cancer's resistance to current treatment modalities. Solid tumors like GBM are known to harbor physical abnormalities at the tissue and cell level. An understanding of GBM cell mechanics may therefore lead to the identification of novel disease mechanisms, biomarkers, and therapeutic strategies to improve treatment outcomes.

In this study, we aimed to compare the mechanics of human GBM cells and their normal counterpart, immortalized human astrocytes (IHAs). To accomplish this, we used a parallel-plate flow system to expose single cells to physiological fluid shear stress and recorded the resulting deformation using a camera-coupled microscope. We then utilized digital image correlation analysis to create strain-time profiles of individual cells and fit the profiles with a three-parameter generalized Maxwell model to characterize their nuclear and cytoplasmic viscoelastic properties. We also used fluorescence microscopy and atomic force microscopy to quantify actin density and alignment and adhesion energy in both cell types, respectively.

Our results showed that, contrary to extracranial cancer cells, the viscoelastic properties of GBM cells are similar to their normal counterpart. This may explain why metastasis in GBM patients is rare and conventional treatment options are unsuccessful. We also found that actin organization and localization and adhesion energy is different between GBM cells and IHAs. Thus, future research which links the genetic and microenvironmental factors that modulate these unique mechanical features may lead to the development of rational and novel clinical strategies against GBM.

Microtubule Dynamics in Bone Metastasis of Breast Cancer

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Bone metastasis (BM) remains a major cause of morbidity in advanced cancer patients. Median survival rates of the two most common BM cancers, breast and prostate, range from 19-25 months and 12-53 months, respectively. Treatment strategies usually rely on surgical resection of tumors, combined with radio- and/or chemotherapies like paclitaxel, which target microtubules (MT). The efficacy of these approaches is diminished by tumor recurrence and drug resistance. Growing evidence points to dysregulated expression of tubulin isotypes as a major driver of chemoresistance. MTs are composed of heterodimers of α- and β-tubulins. Tubulin β3 has been most studied for its resistance to taxanes and vinca alkaloids, with high expression correlating to poor patient outcomes. Tubulin isotypes are distinguished by their c-terminal domain and a variety of post-translational modifications (e.g., acetylation) that facilitate specific protein interaction. Currently, little is known about how microtubules change during bone metastasis. Here, we set out to profile these changes by assessing differences using RNA-Seq data, mass spectrometry, xenograft models, and breast cancer patient samples. RNA-Seq analysis between a set of bone metastatic and a set of non- metastatic breast cancer cell lines (n=10) revealed differential expression of tubulin β isotypes with increased expression for tubulin β2a, β2b, and β6. Protein levels of tubulin β2 were higher in the aggressive MDA-MB-231 compared to MCF7 breast cancer cells. Immunohistochemistry of BM tumors shows increased β3 and acetylated α-tubulin (Ac- αTub) staining compared to matched primary tumors. Ac-α-Tub promotes microtubule stability and supports vesicular trafficking. Together these findings suggest specific microtubular changes during cancer cell adaptation to the bone. We and others have reported that Runx2 promotes bone metastasis. Inhibiting Runx2 in MDA-MB-231 leads to decreased Ac-α-Tub as well as tubulin β2. Mass spectrometry and immunoprecipitations suggest that Runx2 regulates the interaction of MT-associated proteins and HDAC6 with α-tubulin, preventing its deacetylase activity. Loss of Runx2 sensitized these bone metastatic cells to docetaxel and vinblastine treatment, as well as reduced secretion of IL-6. In summary, our results suggest specific changes in microtubule cytoskeleton during BM, as well as novel therapeutic targets that may lead to more effective chemotherapeutics to inhibit tumor growth in bone.

Optimization of a machine-learning-based approach to quantify and characterize microtubule dynamic instability

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Microtubules (MTs) are biological polymers that constitute an essential part of the cytoskeleton of eukaryotic cells and are found in all characterized eukaryotic organisms. MTs display

"dynamic instability" (DI), a behavior traditionally characterized by stochastic switching between phases of growth and shrinkage separated by transitions called catastrophe and rescue. This behavior requires energy, is intrinsic to the MT subunits (it does not require additional proteins) and is fundamental to MT functions. To understand how MT dynamics are regulated, experimentalists must characterize and quantify DI behavior. Classical approaches assumed that DI has only two phases, with instantaneous transitions between them, but high-resolution experimental data shows that DI is more complicated. To address this problem, we developed the Statistical Tool for Automated Dynamic Instability Analysis (STADIA), which uses unsupervised machine-learning approaches to characterize and quantity DI behaviors (including the "stutters" that frequently occur before catastrophe) using microtubule length-history data as input (Mahserejian et al., 2022). Our first release of the STADIA software serves as an improvement over traditional DI analysis methods and provides a foundation for further refinement. The purpose of this project is to improve STADIA by refining its methods for segmenting the inputted length-history data and classifying the line segments. We propose to use a change-point detection-based method to improve vertex selection in the segmentation stage and an advanced clustering algorithm to optimize the methodology used for classification. These changes will further refine the classification and quantification of DI, providing a more comprehensive and data-driven approach to analyzing MT dynamics compared to previously developed techniques.

Robust dynamics of vimentin intermediate filaments visualized using SunTag labeling

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Intermediate filaments (IFs) are integral components of the cytoskeleton, traditionally considered as static 'mechanical cores' in eukaryotic cells. Previous studies have demonstrated the transport of vimentin intermediate filaments (VIFs) along microtubules, facilitated by the kinesin-1 motor, using the photoconversion technique. In this study, we reveal the active transport of VIFs globally within the cell. By employing the SunTag labeling approach, we generated bright and photostable dots on individual VIF, enabling precise tracking of their dynamics for extended durations.

Vimentin-SunTag live cell imaging unveiled the dynamic nature of VIFs throughout the entire cell. Moreover, we observed bidirectional transport of VIFs along microtubule tracks, a process dependent on the coordinated activity of microtubule motors, particularly kinesin-1 and cytoplasmic dynein. Intriguingly, within VIF bundles, individual Vimentin-SunTag dots often exhibited transport in opposite directions. This unique behavior suggests that individual filaments within a vimentin bundle are not interlinked, a finding corroborated by our high-resolution 3D Focused Ion Beam Scanning Electron Microscopy imaging. The vimentin-SunTag labeling approach opens avenues for a deeper understanding of the biological significance of vimentin intermediate filament dynamics and their role in various cellular processes.

Zyxin Recognizes Strained Actin at Focal Adhesions

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The actin cytoskeleton and its associated adhesion and motor proteins are key regulators of mechanical signaling in cells. These structures not only enable cells to generate and transmit forces to the extracellular environment, they can also act as mechanosensitive scaffolds for proteins that sense and respond to mechanical forces. LIM domain proteins, including zyxin and paxillin, have emerged as cytoskeletal mechanosensors that recognize strained actin through a conserved mechanism requiring their LIM domains. While significant progress has been made in determining how strain is detected by these proteins, we still do not understand how mechanical signals are propagated along stress fibers (SFs) and how they impact the structure and function of the proteins residing in the focal adhesions (FAs) they are connected to. To answer these questions, we combined live cell imaging with laser photoablations to reduce the overall SF tension and simultaneously quantify protein intensity changes over time in the adjacent FAs. Surprisingly, we observed that the intensity of proteins such as kindlin and vinculin remained unchanged, while both paxillin and zyxin intensity was reduced when the tension in the SF dropped. These results suggest that both zyxin and paxillin recognize strained actin in the FA itself and that their localization is tension dependent. We have shown that zyxin and paxillin are excellent predictors of traction stresses using a machine learning approach and attribute this effect in part due to their mechanosensitivity at FAs. Our goal is to test using these same methods whether the predictiveness of other FA proteins, including vinculin and kindlin, are comparable to zyxin and paxillin. Together, these results demonstrate that zyxin and paxillin localization to FAs is dependent in part on SF tension and that FAs contain strain sites themselves. Our hypothesis is that FAs act as mechanosensitive hubs to relay information about tension in the cytoskeleton via LIM domain proteins. Our future goal is to investigate how changes in SF tension affect the intramolecular tension of individual FA proteins using FRET-based approaches.

Interrogating the Role of Lysosomal Tubulation in NPC Disease.

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Niemann-Pick Type C disease (NPC) is a pediatric neurodegenerative disease without an FDA-approved treatment. NPC disease is a lysosomal storage disorder caused by mutations in two genes, NPC1 and NPC2, which are each lysosomal cholesterol binding proteins. Mutations in these genes impair the intracellular transport of cholesterol out of the lysosome, and this defective transport contributes to the neuronal cell loss in NPC disease. Recently, the Vaughan laboratory proposed lysosomal tubulation as one mechanism of cholesterol transport that requires functional NPC genes. Tubulation is significantly reduced in NPC diseased cells. Through proteomic analysis, we have identified StARD9 as a candidate gene involved in tubulation. StARD9 contains a functional kinesin motor domain, and a steroidogenic acute regulatory (START) domain. While START domains are known to bind to specific lipids, it is unclear which lipid binds to the START domain of StARD9. In order to determine this, we have overexpressed, solubilized, and purified the START domain of StARD9 in E. coli. We plan to complete ligand binding assays and crystallography with collaborators to more fully understand the structure of this domain. One candidate ligand is 27-hydroxycholesterol (27-HC). 27-hydroxycholesterol is able to significantly activate tubulation in cells. We propose that a function of the NPC genes is to transport 27-hydroxycholesterol (27-HC) across the lysosomal limiting membrane to stimulate StARD9. Interestingly, 27-HC can also rescue tubulation defects in NPC diseased cells, indicating that exogenous addition of 27-hydroxycholesterol could be therapeutic for the treatment of NPC disease. Currently, we are examining the role of StARD9 in tubulation, and the potential of 27-HC as a therapeutic in mouse models of NPC disease.

Synthetic control of dynamic pattern formation at the cell cortex

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The cell cortex undergoes dramatic remodeling to drive cell shape changes necessary for cell division, motility, and morphogenesis. Cortical dynamics are often regulated by Rho family GTPases, which cycle between an active, GTP-bound state, and an inactive, GDP-bound state. Cycling between Rho's active and inactive state is primarily regulated through Guanine nucleotide exchange factors, or GEFs, which promote activation of Rho via nucleotide exchange, and GTPase activating proteins, or GAPs, which promote Rho inactivation via GTP hydrolysis.

This cycling of Rho generates patterns at the cortex such as patches, rings, or stripes, in addition to propagating, complementary waves of Rho GTPase activity and F-actin disassembly – referred to as cortical excitability. Cortical excitability is subject to both internal and external regulation, including internal signaling which prompts cell cycle progression and development.

We have previously shown that cortical excitability can be induced in *Xenopus* oocytes (which are not normally excitable) via co-expression of two Rho regulators essential for cytokinesis: Ect2 (a GEF) and RGA-3/4 (a GAP). To further investigate this excitable circuit, and cortical excitability overall, I have used a synthetic biology approach. Specifically, I employed a synthetic GEF construct to provide Rho-based positive feedback by utilizing the Rho GEF domain of LARG fused with rGBD, a domain that binds specifically to active Rho. When expressed with RGA-3/4, a natural participant in Rho-based negative feedback, the synthetic positive feedback construct, *Rho Positive Feedback 1 (RhoP1)*, produces "semi-synthetic waves" of Rho activity and F-actin. The waves are elongated and propagate slowly, with a relative amplitude of 1.18 and period of 436 seconds, compared with non-synthetic waves produced via co-expression of the two natural excitability regulators, Ect2 and RGA-3/4 (relative amplitude: 0.82; period: 152 sec). Relative to Ect2, expression of *RhoP1* results in heightened cortical contractility. Similarly, a synthetic inhibitor, dubbed *Rho Negative Feedback 1 (RhoN1),* containing a Rho GAP domain and tropomyosin (to bind F-actin), supports semi-synthetic, pulsatile waves when co-expressed with Ect2. These waves show a relative amplitude of 0.17 and period of 320 seconds. Interestingly, co-expression *RhoP1* and *RhoN1* produce fully synthetic, semi-stable patterns of extremely high amplitude. Use of other synthetic inhibitors alongside Ect2 alters resulting wave period and morphology. Further study of this system and development of additional synthetic constructs designed to interact with Rho, F-actin, or their regulators will provide further information about excitable dynamics of this circuit.

ABSTRACT

The Hexosamine Biosynthetic Pathway is a key regulator of cell adhesion and proliferation in metastatic prostate cancer

Rajina Shakya*, Praveen Suraneni*, Dileep Varma
* shared credit, underlined - presenting

Metabolic reprogramming is an important hallmark of tumorigenesis with cancer specific metabolic changes being tightly correlated to cell proliferation, and metastasis. However, the mechanism of how these cellular changes are manifested downstream of the metabolic programs in normal and transformed cells have largely not known. The hexosamine biosynthetic pathway (HBP) is one such key glyco-metabolism pathways resulting in the production of UDP-N-Acetylglucosamine (UDP-GlcNAc) which mediates N- and O-glycosylation of intracellular proteins. The enzyme, GNPNAT1, is an essential rate-limiting enzyme of the HBP pathway. This pathway is essential for regulating metabolic activities and maintaining cellular homeostasis. Recent studies suggest that castration-resistant prostate cancer (CRPC) cells become more proliferative and aggressive when the HBP pathway is inhibited, although the specific mechanism underlying is still unclear. It has been demonstrated that substantially reduced GNPNAT1 was expressed in CRPC than in normal prostate cells. In this study, we have developed CRISPR/Cas9-mediated GNPNAT1 knockout (KO) models in human CRPC cells and normal prostate cells to investigate how the HBP pathway affects the metabolic and transcriptional profiles in these models to govern the cell proliferation and metastatic properties. We hypothesize that changes in these profiles alter the cytoskeletal framework of cells to govern the downstream cellular phenotypes. We find that GNPNAT1 inhibition in CRPC cells indeed induced the above-mentioned altered cellular phenotypes of increased cell proliferation and loss of cell adhesion. Our transcriptomic, metabolomic, and glycoproteomic analyses revealed alteration in several genes, downstream metabolites, and glycoproteins in GNPNAT1 KO CRPC cells. Based on these results, we have proceeded with additional experiments to continue exploring the role of HBP in cellular functions. We demonstrate that GNPNAT1 inhibition drastically alters mitosis, and mitotic spindle morphology, causing chromosomal mis-segregation by elevating microtubule associated protein, MAP10, which controls microtubule integrity, and by downregulating spindle assembly checkpoint protein, BUB1. Additionally, GNPNAT1 inhibition was observed to activate the PI3K/AKT downstream signalling pathways including mTOR, phosphokinase C, FOXO3a transcription to induce cell proliferation. We also observe that GNPNAT1 inhibition affect cellular homeostasis by suppressing YAP phosphorylation and accelerating YAP/TAZ complex nuclear translocation, turning off Hippo signalling pathway. GNPNAT1 inhibition impairs cell adhesion by the mislocalization of EphB6 and affecting MAPK/ERK signalling which in turn promotes cell migration by affecting RhoA/RND3 pathway and Epithelial to Mesenchymal Transition (EMT) transition. Our results suggest that the HBP is a critical component for metabolic control of cancer cell behaviour and that targeting this pathway may serve as a novel strategy for treating CRPC.

ACTOMYOSIN CONTRACTILITY IN THE FORMATION AND FUNCTION OF THE AXON INITIAL SEGMENT

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In addition to electrochemical and biochemical communication, neurons are also thought to communicate via mechanotransduction. Decades of research has established that neurons sense their mechanical environment during differentiation, pathfinding, and pathological perturbations, such as traumatic brain injury or plaque deposition. However, the molecular mechanism(s) by which mechanical cues are generated, sensed, and interpreted by neurons remain incomplete. Myosin 2 is the dominant contractile motor protein in cells, and individual myosin 2 monomers assemble into filaments that pull on the actin cytoskeleton to drive contraction events. Consistent with recent literature, my preliminary data demonstrates a significant increase in active phosphorylated myosin 2 in a subcellular domain at the proximal base of axons, named the Axon Initial Segment (AIS). In addition to myosin, a mature AIS also contains the master scaffold protein Ankyrin-G that anchors voltage-gated ion channels, signaling proteins, and cell adhesion molecules to the neuronal cytoskeleton. Therefore, the AIS contains all the requisite components of adhesion-mediated mechanosensation: active actomyosin with transmembrane components that can couple the extracellular matrix to the intracellular cytoskeleton. The proximal spatial location of the AIS to the soma and nucleus makes it an opportune candidate for transducing mechanical cues into electrochemical and biochemical information that modulate neuronal decision making and behavior. We are currently using traction force microscopy (TFM) to characterize AIS mechanics. In addition, we hope to delineate the spatiotemporal regulation of myosin filament assembly more precisely in the AIS. Our overarching hypothesis is that actomyosin-generated contractility is critical for AIS maturation and neuronal mechanosensation.

Cell Metabolism Modulates Contractility by Regulating Actomyosin Assembly and Signalling at Adhesions

Lee D. Troughton, Stefano Sala Jordan R. Beach, and Patrick W. Oakes

Tissue morphogenesis and homeostasis are dynamic processes that require controlled cell migration and extensive extracellular-matrix remodelling. These processes rely heavily on ATP produced through various cellular metabolic pathways. A key player in these processes is the actomyosin cytoskeleton, which requires ATP for contraction and for optimal filament assembly. As such, the availability of ATP is likely to tune the underlying mechanical interactions and downstream signalling via mechanotransduction. Surprisingly, how metabolic activities affect the cellular contractile machinery and the rest of the cytoskeleton remains unclear. Here we use both fibroblasts and endothelial monolayers to investigate how relatively small-scale changes to metabolism alter contractile behaviour and downstream signalling via adhesions. Using either 2-deoxy-D-glucose to inhibit glycolysis or AMP to increase metabolic activity, we established a model system enabling us to modulate intracellular ATP. By combining traction force microscopy with fluorescent probes to quantify intracellular ATP levels, we have characterised how tension across cell-matrix and cell-cell adhesions is instantaneously modulated by changes in intracellular ATP concentrations. These observations were independent of changes to calcium flux and phosphorylation status of myosin, suggesting mechanical tension is directly connected to ATP availability. Using indirect-immunofluorescence and western blotting to look for alterations to the cytoskeleton during these metabolic fluctuations, we reveal dramatic changes to actomyosin structure and filament assembly rates, while vimentin intermediate filaments and microtubules remain unaltered. Changes in the actomyosin cytoskeleton were also coupled with changes in signalling at focal adhesions. Finally, using RT-qPCR we demonstrate that changes in cellular metabolism modulate downstream gene expression of key players involved in ECM remodelling, thereby regulating tissue morphogenesis and homeostasis. Collectively, our data reveal both rapid and prolonged metabolic modulation of mechanical machinery, eliciting a wealth of questions throughout cell and tissue physiology and pathophysiology.

Gαq homeostasis is required for achieving the optimal organ shape and size through JAK/STAT signaling and P-myosin contractility in developing *Drosophila* wing disc.

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Keywords: Calcium signaling, Ca^2+, growth control, Drosophila wing disc, G alpha q, Gαq, transport, actomyosin contractility

Abstract

Gαq, the G protein alpha subunit of various GPCRs, is used during the development for conveying many external signals into the growing cell. Gαq is able to activate both Ca^{2+} signaling and PKC signaling, for organ to achieve the required size and proper shape, and to regenerate, if needed, the damaged tissue. Gαq is important for human health because mutations in GNAQ (Gαq human ortholog) are implemented in Weber-Sturges congenital syndrome and uveal melanoma. Since Gαq plays role in the initiation of the pathway, the following disturbance of other members of the pathway, in turn, can also cause various serious diseases and cancers. However, little is known about the downstream effectors of Gαq, and its mechanism of action during the development.

Genetically downregulated Gaq signaling during the wing development causes a small and un-spread wing phenotype, and Gaq upregulation causes normal but small wing. Using genetic perturbations, phenotypic wing assessment, wing disc immunohistochemistry and GCamp6f calcium sensor we first confirmed the phenotypes of Gαq perturbations and its relation to the calcium activity in wing discs. Next by employing RNAseq we found that JAK/STAT signaling pathway was affected most when Gaq signaling was perturbed. We also found that Gαq KD and Gαq OE causes cytoskeletal changes in wing discs and we explained the Gaq KD un-spread wing phenotype with dysregulation in transport function and loss of P-Myosin contractility.

In summary, Gαq homeostasis is required for achieving the optimal organ shape and size through activation of Calcium and PKC signaling, JAK/STAT signaling pathway and proper actomyosin localization and contractility.

Septins, Myosin18Aα, and MRCK form a distinct network of stress fibers under the nucleus

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Septins are a family of GTP binding proteins that form hetero oligomers to build higher-order structures in the cytoskeleton. While the function(s) of these higher-order structures remains elusive in many cell types, septins are suggested to act as protein scaffolds and diffusion barriers. Septins can directly bind actin, microtubules and curved membrane, providing a diverse range of potential capabilities and cell processes in which they might contribute. Therefore, understanding septin localization, architecture, and function in different cell types is of great interest. Our fixed imaging of septin in mouse embryonic fibroblasts revealed several distinct populations; the most striking of these being cortical and perinuclear septin fibers. Fluorescent recovery after photobleaching (FRAP) of these distinct septin pools demonstrates that subnuclear septin fibers are more stable than cortical septin fibers. This may be due to architecture, composition, or membrane association. To understand the architecture of septin filaments we used SIM and iPALM imaging. Both cortical and subnuclear septin fibers appear in in a layer below actomyosin with a gap between the majority of septin and actin, suggesting there may be other proteins involved in maintain this network, though its function remains unclear. In looking for other members of this complex we turned to Myo18Aα, and its upstream effector MRCK as these have been observed in similar subnuclear structures. Fix and stains show strong co-localization of septin and myosin18Aα/MRCK, pointing towards a relationship that may facilitate the maintenance of these septin networks. Finally, to understand the role the nucleus may play in directly recruiting septin we disrupted the LINC complex and we observe that septin was not organized but still accumulates under the nucleus, pointing towards signaling beyond the LINC complex to recruit septin. We used glass beads to mimic the biophysical cues the nucleus may be providing and found that glass beads do cause recruitment of septin filaments. To limit the biochemical signals of the nucleus that may still exsit and allow the beads to recruit septin we enucleated cells. Cells that have been enucleated broadly seem to lack significant septin filaments. Our next steps will combine use of the beads and small molecules in the enucleated cells to elucidate what factors that contribute to septin network formation.

DISCOVERY OF A NOVEL SWITCH IN CYTOSKELETAL MICROTUBULE ISOTYPES DURING OSTEOBLAST DIFFERENTIATION

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INTRODUCTION

Bone development relies primarily on the differentiation of osteoblasts into osteocytes, making up over 90% of cells in bone tissue. As osteoblasts differentiate, they undergo remarkable changes in gene expression leading to secretion of proteins and mineralization. These processes rely on a network of microtubules (MTs) to support intracellular vesicular trafficking. Here, we aim to better understand the specific changes occurring in MTs during osteoblast differentiation. MTs are cylindrical structures composed of heterodimers of α- and β-tubulin. Their function is influenced in part by post-translational modifications, one of the most studied being acetylation of α-tubulin, which acts to promote MT stability. An understudied aspect of MTs are the different isotypes of α- and β-tubulins that are integrated into its polymer structure. These and other factors that control the properties and "language" of MTs are known as the Tubulin Code.

METHODS

Two cell lines were used to model bone differentiation, the mouse IDG-SW3 and fetal human osteoblast (FHOBs). Downstream analysis employed alizarin red staining, western blots, qPCR, and immunofluorescence. Publicly available ChIP-Seq data was used to assess differentiating MC3T3. Immunohistochemistry (IHC) analysis was further performed on C57BL/6 mouse femurs.

RESULTS

Differentiation of osteoblasts alters the expression MT isotypes, most notably an increase of TUBB2 and decrease of TUBB3. Both mouse and human osteoblasts demonstrate a sharp drop of TUBB3, whereas differences of TUBB2 are more apparent in the mouse IDG-SW3. This may be explained by a predominant nuclear localization of TUBB2 in FHOBs as shown with immunofluorescence. IDG-SW3 also exhibits differential staining but within the cytoplasm. ChIP-seq analysis of differentiating MC3T3s revealed the recruitment of Runx2 to the TUBB2 gene. IHC of mouse femurs also demonstrates differential staining of these isotypes within the endosteum.

CONCLUSIONS

Our study demonstrates a novel switch of TUBB2 and TUBB3 expression during bone development. Increased TUBB2 we believe reflects an increase in MT stability to promote the demands of bone mineralization. A key regulator of this switch appears to be Runx2 via its binding to the promoter of TUBB2. These findings and a better understanding of the Tubulin Code may one day serve to improve targeted therapies for bone diseases.

The HOPS Complex is essential for endolysosomal transport in axons

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Autophagy is crucial in neurons and disruptions to autophagic flux are implicated in numerous degenerative disorders. Autophagy in neurons is unique. These cells require autophagic induction in the distal axon and subsequent vesicle fusion steps during retrograde transport back to the cell body for full lysosomal degradation of cargo. The coordination of vesicle fusion and transport is essential for efficient transport of autophagocytosed cargos in the axon. In yeast and mammalian cells, the HOPS complex is essential for fusion of late endosomes and autophagosomes with lysosomes or, in yeast, the vacuole. This complex is composed of six subunits, including the core protein Vps18 and the Rab-binding protein Vps41. Disruption of HOPS-mediated vesicle fusion results in impaired autophagic flux in cultured cells and mutations in Vps41 are causal in neurodegeneration with ataxia and dystonia. We investigated the cellular function of HOPS in the endolysosomal pathway in vivo in neurons of larval zebrafish. Loss of either Vps18 or Vps41 caused severe loss of all endolysomal components from the axon. Specifically, early and late endosomes, autophagosomes, and lysosomes were largely absent from the axon. Axon terminal autophagosome populations were dysmorphic and showed large scale aggregation but cell body populations were unaffected. Disrupted acidification of autolysosomes in the axon is associated with impaired transport which can lead to aggregation. We reasoned that loss of axonal organelles could be due to failed acidification from impaired fusion. Analysis of acidified lysosomes revealed a significant reduction of acidifying organelles in HOPS mutants. Furthermore, disrupting autophagic progression pharmacologically recapitulated HOPS mutant phenotypes. Together, these data argue that HOPS is essential for maturation of endolysosomal components in the axon which is required for the formation and transport of organelles in the autophagosome-autolysosome-lysosome pathway.

Capping Protein contributes to the balance of actin assembly among different F-actin networks in the *C. elegans* zygote

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The actin cytoskeleton consists of actin and actin-binding proteins, which organize into filamentous (F)-actin networks with diverse morphologies and dynamics to accomplish a diverse range of cellular tasks. These processes often occur simultaneously within a single cell—so how do cells self-organize such diverse structures from a single pool of proteins?

The cortical actin cytoskeleton in the *C. elegans* zygote undergoes rapid changes during the first cell division of development. During late interphase this cell assembles filopodia, which are membrane-bound bundles of actin assembled by two different actin assembly factors: Formin, a processive elongator, at the tips, and Arp2/3 complex, a nucleator that makes branched F-actin networks, at the base. During mitosis, the cell transitions to assembling mini-comets, which are assembled by Arp2/3 complex. Both the filopodia and the mini-comets contain Capping Protein (CP). CP binds the barbed end of a filament and prevents further elongation and is thus a regulator of actin filament length. What is CP's role in the assembly of these two distinct networks?

Using near-TIRF microscopy, we find that knocking down CP via RNAi in the zygote causes overassembly of filopodia and underassembly of mini-comets, indicating a role for CP in maintaining the balance of assembly among these distinct networks. Additionally, CP RNAi produces longer-lived filopodia, suggesting a role for CP in the turnover of filopodia, consistent with evidence that CP and Formin antagonize each other's residence on barbed ends. We hypothesize that overassembly of filopodia causes underassembly of mini-comets by mis-allocating the limiting pool of actin monomers toward filopodia instead of mini-comets. We are currently testing this hypothesis using a temperature-sensitive formin in cells treated with CP RNAi. We predict that perturbing formin, which is normally not involved in comet assembly, will depress the overassembly of filopodia and thus partially rescue comet assembly in CP knockdown conditions.

Using single-molecule techniques to compare binding kinetics of CP *in vivo* in the zygote and *in vitro* with purified *C. elegans* proteins, we discovered a surprising paradox: the lifetime of CP-barbed end binding events *in vivo* is two orders of magnitude shorter than *in vitro*. This suggests the presence of CP regulators *in vivo,* which may change CP on- or off-rates in different networks to achieve different filament lengths. We are currently investigating the roles of putative CP regulators to understand how CP on-rates may be regulated *in vivo*, and how differential regulation of CP may contribute to the diversity of distinct F-actin networks.