Abstract

Cell mechanics play a central role in motility, cytokinesis, and tissue architecture. The force-generating multimeric protein non-muscle myosin-II is a major player in the mechanics of both healthy and diseased cells, including those involved in certain cancers. Here, we explore factors that govern the assembly of myosin-II, explore its force dependence, and demonstrate that 14-3-3 proteins, which are highly conserved across phyla, are novel regulators of myosin-II assembly. Through a series of biochemical assays, we show that these proteins interact directly, with high affinity, and independently of phosphorylation. Furthermore, we show that both human and amoeboid myosin-IIs are tuned by their respective 14-3-3s, demonstrating evolutionary conservation of this pathway. These findings provide a means to integrate the observed shifts in 14-3-3 expression patterns with the prominent role of myosin-II in tumorigenesis.

The 14-3-3 family comprises a group of small proteins that are essential, ubiquitous, and highly conserved across eukaryotes. Overexpression of the 14-3-3s sigma, epsilon, zeta, and eta correlates with high metastatic potential in multiple cancer types. Through studies in *Dictyostelium* (one 14-3-3, one myosin-II) and humans (seven 14-3-3s, three non-muscle myosin-IIs), we have uncovered the mechanism for myosin-II assembly regulation by 14-3-3s. In *Dictyostelium*, 14-3-3 promotes myosin-II turnover in the cell cortex and modulates cortical tension, cell shape, and cytokinesis. Here, *in vitro* assembly assays using purified myosin-II tail fragments and 14-3-3 demonstrate that this interaction is direct, phosphorylation-independent, and has a high effective affinity ($K_D$...
~300 nM_dimer). All seven human 14-3-3s also affect the assembly of myosin-IlIs to varying extents. Our findings demonstrate a novel mechanism for regulating myosin-II assembly that is mechanistically conserved across a billion years of evolution from amoebas to humans. We predict that altered 14-3-3 expression in humans inhibits the tumor suppressor myosin-II, contributing to the changes in cell mechanics observed in metastatic cancers.

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Chapter 1. Introduction

Cytokinesis has been appreciated as being a complex mechanical process ever since the early days of Ray Rappaport’s research into cytokinesis mechanisms (8). Rappaport's interest in cytokinesis mechanics was shared by several of his contemporaries, such as Hiramoto (9-11), Mitchison and Swann (12), and Wolpert (13). Their early experiments focused heavily on defining the changes in the cortex which were associated with furrow formation and ingestion (14-16). These studies drew upon several state of the art methods of the day including reshaping the cells mechanically, micropipette aspiration, compression between plates, and force measurement by observing the bending of glass needles. Many of these methods appeared to go out of fashion only to return in more recent years as very effective techniques to assess these cortical changes and monitor cortical mechanics, especially when combined with modern imaging methods. Of the many classical experiments that Rappaport performed, a particularly elegant example was his direct measurement of the amount of force that the cleavage furrows of echinoderm eggs could generate during furrow ingestion. To make these measurements, Rappaport inserted two glass needles into the dividing egg, one of which held the cell in place and allowed the second, softer needle to deflect in response to applied forces. Then, by examining the bending of the needle, Rappaport determined the amount of force (~20-30 nN) that the cleavage furrow could generate (17). Rappaport pointed out that these measured forces only revealed what the furrow was capable of generating and that the system was likely to be highly dynamic, adjusting its force generation as needed. Indeed, our current
understanding is that the contractility machinery is regulated not only through forward biochemical pathways, but also through mechanical feedback loops to ensure appropriate levels of force generation and preserve cytokinesis fidelity.

Dissecting cortical changes during cytokinesis reveals three major modules whose mechanical features complement each other in order to ensure robust progression through cytokinesis. The first module is the cortical meshwork of cross-linked actin, which defines local mechanics and the energy cost for deformation. The second module is myosin II, which can be distributed around the cell to generate stress and alter local viscoelasticity, and which is itself mechanoresponsive, accumulating to sites of stress. Finally, a control system of regulatory proteins feeds into the other two modules, tuning their outputs to drive cell rounding, position the equator, and then generate and sustain cleavage furrow ingression in a highly stereotypical manner.

**The cell cortex**

The main goal of cytokinesis is for the cell to remodel its shape until a major topological change occurs in which a mother cell splits into two daughters. The majority of this remodeling occurs at the cell cortex, the cell’s "skin", which consists of the plasma membrane and the underlying cytoskeletal polymer network (5, 18). The cortex of a living cell is a complex, dynamic environment, which is called upon to perform a far-reaching array of cellular tasks. The cortex must be strong enough to withstand stresses imposed by mechanical insults, osmotic pressure, and cell-to-cell adhesions, yet flexible enough to accommodate a broad range of cellular morphologies, from spherical oocytes to flattened epithelial cells to elongated neurons, as well as adaptable enough to
actively remodel itself in response to signals. To accommodate these numerous mechanical demands, the cortex relies on several basic building blocks, each with their own unique time-scale of association. The actin filaments provide mechanical connections between different parts of the cell, and cross-linking proteins join these actin filaments to each other and to the cell membrane. Actin polymer dynamics along with myosin II interactions aid in network remodeling (19-21).

The features of the cytoskeletal network define how the cell will deform in response to applied mechanical stress, whether internally generated or externally imposed. The cellular cytoskeleton is described mechanically as being viscoelastic, meaning that the cortex has both elastic and viscous character (22-24). Rheological measurements reveal that on short time-scales (<0.2 s) the cell cortex has a mechanical phasing of ~15°, which indicates that elasticity dominates at these time-scales (a pure solid has a phasing of 0°, while a pure liquid has a phasing of 90°). On time-scales longer than 0.2 s, super-diffusive behaviors (activities that operate faster than pure thermal motion would allow) become prominent (21). The passive mechanical behavior of the cell is largely defined by the actin network, as latrunculin treatments reduce the total polymeric actin levels by 55%, which causes an 85% reduction in cortical viscoelasticity (24, 25). This non-linear relationship between viscoelasticity and polymer concentration is expected, as pure actin networks and crosslinked actin networks typically have viscoelastic moduli that depend on the actin concentration \( c_A \gamma \) where \( \gamma \) is the power law and typically ranges from >1 to 2.5. The exact value depends on
variables such as actin polymer length, the nature of the crosslinkers, and crosslinker density (26-29).

In cells, the actin polymer length is typically much lower than the persistence length (~10 µm (30)) of the actin filaments. Measured lengths depend on species, ranging from 600-1,000 nm in S. pombe contractile rings to 100 nm in Dictyostelium discoideum cleavage furrows to 45-90 nm in the cleavage furrows of HeLa cells (5, 31, 32). As a result, the filaments behave as rigid rods in the cell and the actin crosslinkers have an essential role in integrating the polymers into a viscoelastic network that can span the circumference of the cleavage furrow. Indeed, multiple actin crosslinking proteins contribute significantly to the spatial mechanics of dividing cells (e.g. global vs. equatorial) and interphase cells (e.g. pseudopod vs. rear end of cell) (5, 24, 33-36). Crosslinking proteins are far from generic, with each having its own unique time-scale for actin association. This leads to time-scale dependent variations in cell mechanics. For example, fimbrin is a crosslinker that associates only transiently with actin, demonstrating a fluorescence recovery after photobleaching (FRAP) recovery time of 260 ms, which is not much slower than the 150 ms recovery time of freely diffusing GFP. Mechanically, fimbrin null cells have a significant reduction in cortical viscoelasticity on the sub-100 ms time-scale but little change in cortical tension, which is measured on the seconds time-scale. In contrast, other actin crosslinking proteins, such as dynacortin and cortexillin I, show impact on sub-second to seconds-time-scale mechanics, along with recovery times (as determined by FRAP) of >0.5 s (5).
The cell cortex may be deformed by stretching ($S_c$) or bending ($B$). The stretch and bending moduli constitute the energy costs for deforming the cortex away from equilibrium. The contribution of each parameter decays over a characteristic length-scale ($l$). Bending ($B$) decays as $l^{-3}$, while stretch ($S_c$) decays as $l^{-1}$. Therefore, as the distance between two points on the surface of the cell increases, the energy costs for deformation can transition between a zone dominated by bending (for small-scale structures) to one primarily influenced by stretch (for larger-scale structures). Based on measured values for these parameters, this cross-over distance is on the order of 100 nm for *Dictyostelium* cells. Since structures such as pseudopods, ruffles, and the cleavage furrow deform cells over several µm$^2$, well beyond the cross-over distance, this implies that the energy cost for stretch will dominate in these cases (5, 34, 37). This dominance is likely to be the case for most cell types and large-scale cortical movements. In fact, the bending modulus has been proposed to decrease at the cleavage furrow of *C. elegans* embryos (38), perhaps to shift the dominant mechanics to a stretch mode within the extremely narrow furrow region which is typical of these cells.

The combination of elastic, viscous, and super-diffusive elements defines the mechanical context under which stresses act at the cortex to drive and guide shape change. The passive stretch coefficient, $S_c$, combines with active stresses to give rise to the effective cortical tension ($T$), which is a major determinant of cytokinesis progression. The cortical tension ($T = \gamma + S_c(A-A_0)/A_0$) is composed of the persistent tension $\gamma$ (which includes the passive tension in the network counterbalancing the osmotic pressure within the cell, as well as active stresses from myosin motors and actin
polymer assembly), and the deformation of the elastic element of the cortex $S_c(A-A_0)/A_0$ (37, 39). Similar formulations for cortical tension have also been described (40). However, it is important to note that the elasticity of the cortex depends on time-scale: at short time-scales the cortex is mostly elastic and resists deformation, whereas at longer time-scales the cortex has more viscous behavior and the effective cortical tension approaches the persistent tension $\gamma$. Thus, cortical tension is a prominent mechanical feature of cells, which combines with local surface curvature to create fluid pressures that serve to minimize the surface area to volume ratio. During cytokinesis, cortical tension first serves to resist cellular deformation, promoting rounding, and then becomes a major driver of cytokinesis furrow ingression once the cell has passed a critical threshold. Cortical tension, when combined with the long time-scale viscous character of the cell, accounts for the kinetics of furrow ingression (7, 41). In addition, cortical tension is predictive of the forces required to drive furrow ingression (42, 43), the molecular requirements for cytokinesis, including how cells can divide without myosin II (7, 41), and the consequences of pressure imbalances, which can lead to cellular oscillations during mammalian furrow ingression (44).

Rappaport considered whether tension gradients were enough to drive furrow ingression, but concluded that they were insufficient (45). A new computational model has also addressed this question by determining the relative contributions of tension gradients to cleavage furrow ingression (41). The authors found that tension differentials could be sufficient for driving cytokinesis; however, the differentials had to be non-physiologically large, consistent with Rappaport’s findings. The model describes
the shape changes of cytokinesis furrow ingression by summing the forces acting around the cell (such as protrusive forces, contractility, and cortical tension), and then finding the energetic minimum around the cell perimeter, thus describing the contours of the cell membrane. While this approach relies solely on experimentally determined values for the types and magnitude of forces and the viscoelasticity of the cell, the actual shape of the simulated cell is unconstrained. The modeled cell closely resembles a dividing cell \textit{in vivo}, and adjustments to the model to eliminate adhesive forces or contractility (\textit{in silico} analogs to cell division on a non-adherent substrate or the division of cells lacking myosin II) also demonstrate the same quantitative behavior as those observed \textit{in vivo} (Fig. 1). The model shows that while cortical tension gradients are major drivers of furrow ingression, they do not act alone; force contributions from protrusion coupled with adhesion and/or myosin II contractility are required to initiate furrow ingression.
Non-muscle myosin II

Myosin II is a significant contributor to cell mechanics, but it does so in complex ways. Myosin II generates contractile stresses on the actin network, which can lead to increased cortical tension and/or relative movement between actin filaments, but the phenotypes arising from this stress generation can vary and at times appear counterintuitive. Across the literature, one can find many seemingly contradictory observations of how myosin II affects and/or tunes cell mechanics. Various reports have indicated in turn that myosin II has no effect on cell mechanics (46), increases the fluidity of the cellular network (47), increases cell deformability (48, 49), or decreases deformability (5, 50-52). Our observations indicate that these apparently conflicting findings may be partially reconciled by considering which actin crosslinker(s) or linking proteins are working in conjunction with myosin II, and whether the network is under mechanical stress (5, 21, 53). The time and length scales under measurement can

Figure 1. Cells can complete cytokinesis effectively in the absence of myosin II (adapted from (6))

A) The net force balance from polar relaxation, traction, and equatorial stiffening leads to cleavage furrow ingress in silico

B) In vivo dividing cells lacking myosin II undergo the same series of shape changes in a similar timescale as modeled cells
showcase different aspects of myosin’s function, and finally, the regulatory state of the myosin protein itself can vary depending on cell type or stage of the cell cycle.

Non-muscle myosin-II is a hexameric motor protein comprised of two heavy chains, two essential light chains, and two regulatory light chains, which associate to form the “functional monomer”. This functional monomer consists of a globular head, which contains the actin-binding motif and the ATPase site, a neck region, which contains the essential light chains and the regulatory light chains, and a long coiled-coil tail region, which contains the assembly domain (54). These monomers are capable of regulated assembly via interaction of the tail domains to form bipolar thick filaments (BTFs), the functional units of myosin-II which bind to actin filaments and hydrolyze ATP to generate force in the cell. Myosin-IIs are major players in cell mechanics, acting to guide and drive cellular processes including cytokinesis, cell migration and tissue invasion, and responses to external mechanical cues (55-57). The dynamics of myosin II regulation are quite elaborate, as its force-generating properties are dependent on its ATPase cycle as well as its assembly state. Current models suggest that in mammals, myosin monomers are maintained in an assembly-incompetent state by heavy chain phosphorylation and an association between the unphosphorylated regulatory light chain and the tail (the so-called 10S structure) (58). Upon activation, the myosin undergoes a 10S-6S transition, whereupon the 10S monomer uncurls to form the assembly competent, elongated 6S state. In *Dictyostelium*, myosin II assembly regulation does not appear to involve the 10S-to-6S transition. While regulatory light chain phosphorylation does occur in *Dictyostelium*, it is not involved in assembly and
only results in a slight (3-5-fold) increase in actin-activated ATPase activity (59, 60). Instead, *Dictyostelium* BTF assembly is primarily regulated by myosin heavy chain kinase (MHCK) phosphorylation of the myosin tail at three key threonines (1823, 1833, and 2029). Phosphorylated myosin remains largely unassembled, whereas unphosphorylated myosin over-assembles readily (61). As expected, mutation of these three threonines to aspartic acids (3xAsp, a phosphomimetic myosin tail) leads to severe defects in myosin assembly, and mutation of the threonines to alanine (3xAla, a non-phosphorylatable tail) leads to myosin-II overassembly and to the formation of aggregates *in vivo* (62, 63). Heavy chain phosphorylation also occurs in mammalian myosins, and current research demonstrates that this has many important consequences for its assembly and localization (64-67). In both *Dictyostelium* and higher eukaryotes, BTF assembly occurs through a nucleation-elongation mechanism, where assembly competent monomers form a parallel dimer and then two dimers form an antiparallel tetramer referred to as the "nucleus" (68, 69). Assembly into larger filaments is then thought to proceed by sequential dimer addition to form BTFs.

Since myosin force generation requires its assembly into BTFs and most myosin II is found in the assembly-incompetent monomeric state (70), the cellular distribution of assembly-regulating factors help direct local contractility. For example, myosin heavy chain kinases (MHCKs) are encoded by four genes in *Dictyostelium*, three of which (isoforms A-C) appear to play important roles in cytokinesis. These isoforms are spatially segregated in the cell with MHCKA enriched at the poles and MHCKB and C localized to the cleavage furrow during cytokinesis (71, 72). In contrast, a myosin II
heavy chain phosphatase (a PP2A member) is solely cytoplasmic and requires the Dictyostelium huntingtin protein for full activation (73). Mutant cells lacking the huntingtin protein fail to maintain myosin II at the cleavage furrow. These enzymes (the MHCKs and the phosphatase) primarily maintain the steady state of unassembled myosin II. The steady state level of unassembled myosin is ~80-90% as determined by biochemical fractionation (70), or 50-70% based on immobile fractions from FRAP analysis (3). Thus, cells maintain a substantial free pool of myosin so that they can assemble BTFs wherever they are needed.

By harnessing such contractility regulators to promote localized assembly of myosin into the BTF form, cells can control their cortical properties to direct spatially regulated shape changes. This effect can be observed at the tail end of migrating cells, as well as in the classic equatorial stimulation model for cytokinesis. There are two groups of contractility regulators important for cytokinesis: the chromosomal passenger complex proteins (including INCENP, survivin, and aurora kinase), and centralspindlin (including kinesin-6 and MgcRacGAP) (74-77). MgcRacGAP is found in higher metazoans, but not in Dictyostelium (78). The centralspindlin complex is thought to activate rhoA in the overlying cortex and lead to the activation of ROCK kinase, which in turn phosphorylates myosin II on its regulatory light chain, promoting the 10S-6S transition. The subsequent assembly of myosin increases local cortical tension and contractility, and thus drives furrow ingression.
Mechanosensation

Myosin II exhibits enhanced binding to actin filaments under tension (79), and will accumulate to regions of high stress within the cell (1, 53, 80). This ability to detect and respond to forces is known as mechanosensation. Mechanosensing provides an important mechanism for directing localization of myosin II and is fundamental to a wide range of cellular and tissue functions (29, 81). We have shown that mechanosensitive accumulation plays a central role in cleavage furrow concentration of myosin II (82). This property has also been demonstrated in other myosins such as myosin I, V, and VI (83-85). There are several force-dependent cytoskeletal phenomena which, when taken together, can explain this behavior. First, actin filaments are highly allosteric, making them ideal mediators of cooperative interactions between actin-associated proteins along the polymer. This allostery allows point forces exerted on an actin filament to propagate subunit conformational changes locally (86). Second, when myosin II proceeding along an actin filament is subjected to a resistive force, its duty ratio increases, causing it to lock onto the strained filament (87). The result of these phenomena is that tension on the actin filaments and/or force generated by a bound motor head will stretch the actin filaments, facilitating the binding of additional motor heads nearby (79, 88). This localized binding of motors on an actin filament then promotes further BTF assembly, forming a force-dependent positive feedback loop (25, 53). A recent multi-scale model demonstrates quantitatively how myosin motor forcesensing and cooperativity and the BTF assembly mechanism are integrated to promote local mechanosensitive accumulation of myosin II (25).
When sliding actin filaments, myosin motor proteins harness the energy of ATP hydrolysis (~100 pN•nm) to perform work over the ~8 nm step size of the myosin lever arm (89). As a longer lever arm will increase the myosin II step size (consequently increasing the unloaded motor speed), conservation of energy dictates that the maximum force generated will be proportionately lower. Therefore, a longer lever arm stalls more easily under load, whereas a shorter lever arm will produce more force and be less sensitive to stalling. Stalling is thought to occur when a bound myosin head is in the isometric state between the pre- and the post-stroke configurations. Several lever arm mutants of Dictyostelium myosin II have been cloned and characterized, and they all retain full motor activity with the expected changes in step size and unloaded motor speed (60). Furthermore, when these mutant motors are expressed in cells lacking endogenous myosin II, they demonstrate the expected trends in mechanosensitive accumulation; all mutants show a linear relationship between accumulation and applied force, but the longer lever arm 2xELC mutant is more mechanosensitive at all pressures, and the short lever arm ΔBLCBS mutant is less mechanosensitive at all pressures. Additional support for the isometric stalling model is provided by the S456L uncoupler mutant, which has the same lever arm length as WT myosin II, but which has a defect in the ATPase region that reduces its step size and unloaded filament sliding velocity below the level of the short lever arm (ΔBLCBS) mutant (89). If myosin’s mechanosensitive accumulation to sites of stress was dependent on its motility, the S456L mutant would be expected to have similar characteristics to ΔBLCBS. However, its mechanosensitive
behavior is similar to WT myosin, indicating that lever arm length is the critical parameter, not motor speed or step size.

While stalled myosin thus functions as an effective force sensor, other factors must also be present in order to drive the productive accumulation of myosin to sites of stress. A reservoir of soluble, assembly-competent myosin feeds the growth of stalled BTFs, and the long-lived actin crosslinker cortexillin I forms stable connections between actin filaments, conducting forces through the stressed network. Myosin also demonstrates hetero-cooperativity with cortexillin I through actin, amplifying the accumulation of both proteins; disruption of either protein abolishes the mechanosensitive accumulation of the other. This complex behavior allows the three proteins (actin, cortexillin I, and myosin II) to form a force-dependent feedback controller, which actively stiffens and contracts to counteract external stresses. The mechanosensitive response is independent of the spindle signaling pathway, but requires proper regulation of myosin bipolar thick filament assembly and regulatory light chain phosphorylation (53). Therefore, this mechanosensory system is only part of a larger control system that tunes the total level of myosin II accumulation at the cleavage furrow under diverse mechanical constraints (82).

The cytoskeletal control system

Several proteins traditionally associated with the mitotic spindle also show accumulation at the cleavage furrow at later stages of cytokinesis, including the kinesin-6 protein kif12, inner centromeric protein (INCENP), and Aurora kinase (75, 76). Interestingly, kif12 and INCENP also demonstrate mechanosensitive accumulation to an
aspirating micropipette, even when the spindle is ablated with nocodazole, a microtubule depolymerizing drug (1). This cortical localization of kif12 could involve a similar mechanism as the cortical localization of mammalian kinesin-6 (MKLP1), which localizes to the stem-body region of the midbody independently of its microtubule association (90). Extensive analysis of mutant Dictyostelium strains demonstrated that the cortical localization of kif12 is dependent on the central myosin II-cortexillin I mechanosensor. This ability of the mechanosensor to feed information about the mechanical state of the cell to upstream regulators is referred to as mechanotransduction, and in Dictyostelium is mediated through the protein IQGAP2. Dictyostelium has three IQGAP proteins, IQGAP1 (a.k.a. DGap1), IQGAP2 (a.k.a. GapA), and a more distantly related IQGAP3. IQGAP1 and IQGAP2 are known to bind to cortexillin I (91, 92). The IQGAP1-cortexillin I complex suppresses mechanosensation, whereas IQGAP2 relieves this suppression as well as mediates mechanotransduction to kif12 and INCENP (Fig. 2). An iqgap1/iqgap2 double mutant cell preserves normal myosin-cortexillin mechanosensing, yet kif12 and INCENP no longer accumulate to the cleavage furrow or to an aspirating pipette (1). These double mutant cells still accumulate myosin to the cleavage furrow, but have reduced cytokinesis fidelity. However, when the cells experience additional mechanical stress, the lack of mechanotransduction compromises their ability to accumulate myosin to the cleavage furrow as compared to WT cells. Thus, mechanosensation and mechanotransduction combine to tune the level of myosin accumulation at the cleavage furrow under a wide

15
range of force regimes. This allows cells precise, robust control over their cortical mechanics, ensuring cytokinesis fidelity under a variety of conditions.

Interestingly, the mechanosensitive behavior of myosin II is heavily attenuated during interphase through the action of the small GTPase racE. If WT cells are aspirated at any stage of the cell cycle prior to the onset of anaphase, the mechanosensitive response is undetectable at low pressures, but upon the transition to anaphase myosin will begin to accumulate to the pipette. The exact mechanism of this attenuation is unknown, but racE is known to play a fundamental role in the regulation of actin crosslinker distribution and in the modulation of cleavage furrow ingression (2, 7). By shifting cytoskeletal stresses onto alternate actin crosslinkers, such as dynacortin and enlazin, the effective force experienced by the mechanosensitive cortexillin I crosslinker would be diminished, thus suppressing mechanosensation. Indeed, cells lacking racE are highly mechanosensitive at all stages of the cell cycle, and also exhibit much lower cortical stiffness, with a decrease in the cortical localization of dynacortin and coronin (2, 53). This evidence suggests that a post-metaphase transition in the regulation of racE allows cells to harness the mechanosensitive abilities of myosin to create a force gradient, leading to furrow ingression and the successful completion of cytokinesis.

14-3-3, a cytoskeletal regulator

Previous work in our lab identified an interaction between 14-3-3 and myosin-II in Dictyostelium (93). 14-3-3 proteins are small acidic regulatory proteins that are involved in numerous cellular processes, including cell cycle control, DNA damage repair, apoptosis, and many signaling pathways (94-97). 14-3-3 proteins are also known
to play many other roles in cell division, such as modulating mitotic translation and centralspindlin signaling (98, 99). 14-3-3s act to scaffold, sequester, or change the conformation of their binding partners, and this interaction is often mediated by phosphorylation of the target protein (100). However, many phosphorylation-independent interactions have also been reported, including to glycoprotein Ib, p75NTR-associated cell-death executor (NADE), inositol polyphosphate 5-phosphatase, and the bacterial toxin exoenzyme S (exoS) (101-103). 14-3-3 proteins are essential in all eukaryotes and are highly conserved, both structurally and by sequence. Multiple paralogs of 14-3-3 often exist in a single cell and can form homodimers or heterodimers with each other (104, 105). While their structural similarity confers a large degree of functional overlap to these paralogs, differences in the paralog-specific effects or interactors are also common (100). Therefore, the expression patterns of 14-3-3s, and their relative ability to heterodimerize, can affect a host of regulatory pathways. In fact, altered expression or dysregulation of 14-3-3s is associated with numerous disease states, including cancers and neurological diseases such as Parkinson’s and Alzheimer’s (106-109). Due to 14-3-3’s position at the heart of so many central regulatory networks, it is perhaps unsurprising that its interaction with myosin passed unnoticed for so long.
We found that in Dictyostelium, 14-3-3 serves as an intermediary between microtubules, racE, and myosin, promoting myosin BTF remodeling and helping to maintain proper cortical tension under the regulation of racE. RacE is responsible for the cortical localization of 14-3-3, and 14-3-3 in turn directly associates with myosin to promote BTF turnover. 14-3-3 also tunes overall microtubule length and is required for normal microtubule-cortex interactions. 14-3-3’s localization to the polar cortex and its interaction with myosin, along with its ability to rescue the cytokinesis defects of racE null cells, suggests that it is involved in establishing the tension gradient between the cleavage furrow and the poles (3). 14-3-3 binds directly to Dictyostelium myosin-II and inhibits its assembly into BTFs, which tunes the balance of assembly and disassembly, maintains a dynamic pool of available myosin subunits, and promotes myosin turnover.

Figure 2. Different regulatory regimes in the cleavage furrow and poles create a force imbalance that drives furrow ingression
The myosin II-cortexillin I-IQGAP-kif12-INCENP control system regulates the equatorial cortex. In contrast, the microtubule-racE-14-3-3-myosin II pathway and global crosslinking proteins control the polar mechanics. Note: the dashed arrows and asterisk (*) denote that the interaction between 14-3-3 and microtubules has been characterized in interphase cells, but due to the sparse nature of astral microtubules in Dictyostelium cells, this interaction has not been fully characterized in mitotic cells. Data are compiled from (1-5).
By doing so, 14-3-3 modulates myosin II activity, cortical tension, cell shape control, and cytokinesis.

**Building a robust morphological engine**

The modules described above are the drivers of cell shape change. The cortical network of cross-linked actin defines the deformability of the cell, myosin II alters local stiffness and tension and accumulates to sites of stress, and the control system tunes cortical mechanics and directs myosin II localization and mechanosensitivity to carry out various cellular programs. During cytokinesis, the output of these modules is furrow ingression. Many descriptions of cytokinesis have explained furrow ingression as being driven by myosin contractility through a sarcomeric-like mechanism involving a "purse-string" of actin and myosin II filaments. However, we believe that it is more accurate to describe furrow ingression as being driven by multiple force-generating systems where cortical tension is a prominent one, and myosin II's function is to contribute contractile stress and an increase in cortical tension at the furrow (7, 41). Because cortical stresses are not only driven by myosin but also depend on factors such as actin polymerization, actin crosslinkers, membrane anchoring, surface curvature, and osmotic pressure (which must be resisted by the cortex), the cell can achieve force differentials in many different ways. This principle explains the ability of cells lacking myosin II to perform cytokinesis (7, 41, 110, 111), as well as the ability of certain mammalian cells to perform cytokinesis in the presence of blebbistatin, a myosin inhibitor (112, 113). It also accounts for why myosin II mechanochemistry is not rate-limiting for furrow ingression and why in *Dictyostelium*, furrow ingression rates are inversely related to the length of the myosin II
lever arm (if filament sliding were the primary mechanism for furrow constriction, the longer lever arm mutants would be expected to ingress faster) (1, 5). This fundamental principle has also been implicated in mammalian cells, where a mutant myosin II that has mechanosensitivity but no actin-filament sliding ability is able to support cytokinesis in cultured cells as well as during embryonic development (114).

The various modules, connected through feedback loops, ensure that the appropriate magnitude of mechanical stresses are distributed around the cortex to generate shape changes, which allows furrow ingression to happen under a wide range of contexts. This robust system allows cells to divide with and without adhesion, in the absence of genes which contribute to cortical mechanics, and in the presence of external stresses such as those experienced by cells in tissues. Inputs that emanate from the spindle trigger initial myosin II accumulation and symmetry breaking, and the force-sensitive feedback loop then tunes the accumulation of myosin II as cytokinesis proceeds. Thus, we can now begin to understand how the same cellular machinery can generate sufficient forces for furrow ingression under diverse mechanical contexts.

**Summary and open questions**

Even after decades of research, the process of cytokinesis continues to amaze and puzzle biologists with its flexibility and complex behaviors. Under normal conditions, cytokinesis appears to be a vertically organized process. Signals from the mitotic spindle lead to the asymmetrical distribution of cytoskeletal proteins around the cortex, which in turn shift the force distribution around the cell, causing constriction at the furrow and protrusion at the poles. However, when cells are stressed mechanically,
this seemingly hierarchal process automatically compensates by drawing upon a mechanical feedback system to adjust the force-generating system to complete cytokinesis successfully. This adaptability allows cells to achieve the same cell shape changes in widely divergent mechanical environments and might explain how some asymmetric cell divisions can occur in the apparent absence of the spindle (115, 116). Cells may also automatically "wire around" proteins that play central roles in normal cell division when these components fail to perform their roles, with multiple feedback loops from downstream proteins ensuring that the cell continues to carry out the necessary shape changes for proper division. Cytokinesis is thus a highly robust cellular program, which can use combinatorial synthesis to achieve the same end result through several different cellular modules, many of which are not immediately apparent in unstressed cells.

The feedback control system may account for much of the historical difficulty in determining which proteins are strictly essential for cytokinesis, and which appear to play a role but are not absolutely necessary. It also explains why certain features that are essential, such as the mitotic spindle, are dispensable after a certain point in the division process—once these components have signaled to downstream modules, force-dependent feedback loops ensure that cytokinesis proceeds smoothly without further signaling. Similarly, a feedback mechanism may also explain why furrow formation can be induced by monopolar spindles (117). Finally, by moving beyond the simplistic designators of "essential" or "non-essential", placing genes within related modules should greatly simplify the process of reconciling data from different model systems.
Indeed, what at first may appear to be a highly organism-specific mechanism is likely to be simply the result of directing the same cellular modules in a novel way to achieve the necessary results—dependable, consistent division under the environmental conditions particular to that cell.

Two areas of this modular view of cytokinesis remain unclear: the mechanism of symmetry breaking which initiates the process, and how stresses are propagated through the cortical cytoskeletal network to the mitotic spindle, forming the mechanical linkage that completes the mechanotransduction circuit. These two questions are intertwined, as the presence of feedback loops between the cortex and the spindle suggests that the spindle itself might not be the obligate “top-down” driver of symmetry breaking. This concept is also implied by the “long axis rule”; the fact that the spindle elongates along the long axis of the cell strongly suggests that mechanical elements influence division plane specification (118, 119). Thus, mechanical feedback and stress propagation through the cortex to the spindle may be critical players in this process. Longer term, determining how the mechanical modules described in this review (cross-linked cortical actin, myosin II, and the control system) operate in the context of complex environments, such as tissues, will become particularly important. Tissues offer a radically different mechanical environment from single cells, including additional adhesions to the substrate and to other cells, a more complex mechanical stress environment, and the possibility of mechanical heterogeneity among otherwise identical cells, particularly within tumors. Determining how these features interact with
the core modules described above will have significant impact on cytokinesis research, as well as implications for health-related biology.
Chapter 2. Promoting myosin assembly

Introduction

As discussed above, a clear simple signaling pathway has not been found yet that fully explains how myosin II accumulates at the cleavage furrow cortex. This is undoubtedly due to system redundancy as multiple pathways - mechanical stress and spindle-mediated pathways – can direct myosin II accumulation. Furthermore, myosin II can accumulate in several systems in the absence of the motor domain, apparently independent of actin polymers, and in a manner that depends on the ability to form bipolar thick filaments rather than on a specific targeting sequence within the myosin II tail. However, in these instances, the myosin fails to incorporate tightly in the furrow cortex or only transiently associates before dissipating again (110, 120, 121). These observations argue for other pathways and/or a cortical receptor (R) that can either help target the BTFs or bind the BTFs in the cleavage furrow cortex. 14-3-3 (encoded by a single gene in Dictyostelium) could be part of one such cortical receptor for myosin II (122, 123). In metazoans, anillin is a candidate to be another such cortical receptor (124, 125).

To search for proteins involved in myosin II cleavage furrow cortex accumulation, we leveraged our genetic tools and the 3xAsp mutant myosin II heavy chain. This myosin II mutant has the three critical threonines mutated to aspartic acids, mimicking the phosphorylated myosin heavy chain and driving myosin II towards the disassembled state (70). The 3xAsp myosin II has severely impaired ability to assemble into BTFs in
vitro, remains largely disassembled in vivo, and typically fails to accumulate appreciably at the interphase or cleavage furrow cortices (70, 126). Our experimental design is based on the assumption that myosin II exists in a minimum of four states, the assembly-incompetent monomer ($M_0$, which is largely populated by the phosphorylated wild type or 3xAsp myosin II), the assembly-competent monomer ($M$, which is thought to be largely populated by the unphosphorylated monomer), the $BTF_n$ (cytoplasm) ($BTF$ of size n in the cytoplasm), and $BTF_n$ (cortex) $\cdot R$ (the $BTF$ of size n in the cortex bound by any cortical receptors $R$). In this case, the myosin II cleavage furrow recruitment will look like the following (123):

3xAsp alone is shifted towards $M_0$, and by expressing 3xAsp in a wild type background (WT::3xAsp), the normal myosin II assembly will be altered leading to cytokinesis defects. We subjected this strain to genetic selection and identified suppressors that could restore myosin II on the cortex (122, 127). We tested the effect of these genes on the distribution of 3xAsp expressed in a $myoII$ null background, so that the only myosin II present is 3xAsp. To test the relationship between myosin mechanosensitivity and heavy chain phosphorylation, we also constructed and
examined an engineered myosin with a long lever arm (2xELC) and the 3xAsp tail, to test whether cross-talk between the cooperative binding by the motor and thick filament assembly would suffice to allow greater furrow accumulation of 3xAsp.

**Genetic suppression of 3xAsp**

To establish a system for identifying proteins involved in novel pathways for myosin II cleavage furrow accumulation, we characterized the effects of 3xAsp myosin II on WT myosin II activity. The 3xAsp mutant myosin II has the threonine residues at positions 1823, 1833, and 2029 mutated to aspartic acid residues, mimicking the phosphorylated WT myosin II, which disrupts the ability of this myosin II to assemble into BTFs (70). Compared to the uniform cleavage furrow distribution of WT myosin II, when GFP-3xAsp was expressed along with mCh-WT myosin II, the two proteins co-localized at the cleavage furrow cortex but the myosin II distribution of both proteins was disrupted with irregular, asymmetric accumulation often observed. We also tested whether 3xAsp could accumulate in response to applied stress in the presence of WT myosin II. We found that when tracking 3xAsp myosin II, the mechanosensory responsiveness of the myosin II was disrupted indicating a severe impairment to myosin II dynamics (128). Finally, while it is well characterized that myoII-null cells expressing 3xAsp are completely unable to grow in suspension culture (70), the WT cells expressing 3xAsp from an episomal plasmid were able to survive in shaking culture but with greatly reduced growth rates as compared to WT cells (not shown).

This combination of defects in uniformity of cleavage furrow accumulation, mechanosensitive localization, and growth rate were considered essential for our
experimental design for using genetic selection to identify genes that encode proteins involved in non-mechanosensitive myosin II accumulation. We then stably integrated GFP-3xAsp into the genome of WT cells, creating WT::3xAsp cells. This insertion was integrated randomly. These WT::3xAsp cells displayed greatly reduced growth rates as compared to WT cells.

With the defects in suspension growth, these WT::3xAsp cells were subjected to cDNA library suppression to select for genes that could rescue the WT::3xAsp cytokinesis defects. We recovered 25 independent genes from the selection. One cDNA included the myosin coding sequence spanning nucleotides 4459 to the poly A tail, which is essentially the coding sequence of light meromyosin (LMM) region of the myosin heavy chain tail. However, the only in-frame ATG did not occur until nucleotide 5671. Thus, LMM_{TF} is predicted to encode only the myosin tail fragment (myosin TF), which spans amino acids 1891-2116. The recovery of LMM_{TF} demonstrated that our cDNA library suppression approach could identify genes involved in myosin II function.

Because LMM_{TF} includes WT myosin sequence that spans the three mutated threonines in 3xAsp, it is possible that the cDNA recombined with the 3xAsp, correcting the residues to WT threonines, especially on the time-scale (up to ~100 hours) of a suspension growth assay. However, we did observe LMM_{TF} suppression on faster time frames as well. Nevertheless, we engineered a construct encoding only the myosin TF region, which includes only the third threonine at position 2029. Myosin TF was able to partially rescue growth in suspension (not shown). If this fragment recombined with 3xAsp, restoring position 2029 to a threonine, this would produce 2xAsp (DDT), which
does not rescue myosin II assembly (129). We also expressed and purified the myosin tail fragment and found that it could indeed assemble, or aggregate, at low salt though its properties were quite distinct as compared to larger tail fragments that included the assembly domain (not shown). Overall, we conclude that the myosin TF region provides some suppression of 3xAsp, but we cannot rule out that LMM$_{TF}$ could also provide additional benefit by recombining with the integrated 3xAsp gene.

**Effect of suppressors on 3xAsp during cytokinesis**

We then tested whether the suppressors could promote 3xAsp cleavage furrow localization in the higher-stringency *myoII*:3xAsp cells, which lack all WT myosin. When expressed alone, 3xAsp seldom shows accumulation at the cleavage furrow cortex. Based on our experimental design, if a gene plays a pivotal role in modulating myosin II assembly or targeting myosin II to the cell division site, expression of this suppressor may help drive 3xAsp myosin II dynamics towards cleavage furrow accumulation. We expressed several recapitulated suppressors in *myoII*:3xAsp cells. From our conception of how 14-3-3 modulates myosin II assembly, we also tested the 14-3-3hp, which reduces 14-3-3 expression by 70% and also leads to over-assembled myosin II in an otherwise WT background (122). We then quantified the myosin II levels at the cleavage furrow cortex by measuring the myosin II intensity at the furrow ($I_f$) and polar ($I_p$) cortices and then calculating the intensity ratio ($I_f/I_p$) for each cell. Histograms of the distribution of 3xAsp $I_f/I_p$ ratios were plotted for each cell-line and compared with the empty vector control (**Fig. 3A**). Several plasmids showed an enhancement of the 3xAsp furrow localization with $p$-values less than 0.10. These plasmids included 14-3-3hp,
LMM_{TF}, and actin. Additional plasmids were tested but are beyond the scope of this thesis; for details, refer to Ren et al., 2014 (130).

One way in which the suppressors could rescue 3xAsp myosin II is by promoting assembly of the 3xAsp myosin II into bipolar thick filaments (BTFs). To test this, we performed total internal reflection fluorescence (TIRF) microscopy in order to examine the BTF assembly state of 3xAsp alone and with the suppressors and in comparison to WT BTFs, which are readily visible by TIRF (21, 71). Contrary to expectation, we found that 3xAsp can form some very short filaments and/or aggregations/bundles in the myoII-null cells. Actin did not significantly alter 3xAsp assembly as compared to 3xAsp alone; however, LMM_{TF} and myosin TF increased cortical 3xAsp levels to different extents.

**Characterization of 3xAsp suppressors**

To ascertain the functional state of myosin II in the rmd1hp cells as well as myoII::3xAsp cells rescued by RMD1 and LMM_{TF} expression, we measured the furrow ingression dynamics and symmetry of daughter cell sizes for these cells. As compared to the near-exponential WT furrow ingression dynamics, none of the mutants were able to recover these WT dynamics. The products of cell division are the daughter cells, which for WT cells are highly symmetrical in size. To quantify this, the 2D-cross-sectional area of the daughter cells is measured, and the ratio of the larger cell to the smaller cell is calculated (Fig. 3B). For WT, this ratio is 1.10±0.021 (mean±SEM) whereas for myoII null cells, the ratio is 1.34±0.084. As compared to WT cells, depletion of rmd1 did not alter the daughter cell symmetry despite altering furrow ingression kinetics (Fig. 3E).
Figure 3. 3xAsp suppressors restored 3xAsp cleavage furrow accumulation

A. Expression of 3xAsp suppressors increased furrow accumulation of GFP-3xAsp in myoII null cells. Epifluorescence images of dividing cells at telophase and final bridge stages are shown. Scale bar, 10 µm. Histograms show the 3xAsp fluorescence intensity ratio of furrow/pole (I_f/I_p).

B. Dot plot shows the distributions of area ratios of the large/small daughter cell for all of the same genotypes. The bars represent the medians. The WT class of cells (WT::EV (EV, empty vector), WT::rmd1hp, myoII::3xAsp;LMM_TF, myoII::3xAsp,RMD1, and myoII::2xELC-3xAsp) produce symmetrically sized daughter cells. The myoII null class (myoII null, myoII::3xAsp, and myoII::3xAsp,mCherry) produce highly asymmetrically sized daughter cells. Asterisks denote significance relative to WT::EV (*p<.05 **p<.005).

C. Time series of a dividing cell expressing GFP-2xELC-3xAsp.

D. TIRF images of cells expressing GFP-3xAsp, GFP-2xELC-3xAsp, GFP-3xAsp;LMM_TF, and GFP-WT myosin II.

E. Furrow-thinning dynamics of WT, WT::rmd1hp, myoII::3xAsp with empty vector or mCherry (mCh), the vectors used to express LMM_TF or RMD1, respectively), myoII::3xAsp;LMM_TF, myoII::3xAsp,RMD1 and myoII::2xELC-3xAsp show that only WT cells have the characteristic exponential thinning dynamics. All other cell strains show a transition to more rapid thinning at a relative diameter of 1 (furrow diameter was rescaled by D_r, which is the point when furrow diameter and length are equal (7)).

F. Cartoon depicts two thresholds of myosin II activity. Daughter cell symmetry requires intermediate level of function. WT furrow ingression and daughter cell symmetry requires the highest level of function. The myoII null scenario has altered ingression dynamics and produces highly asymmetrically sized daughter cells.
Interestingly, RMD1 and LMM$_{TF}$ expression did not improve the furrow ingression kinetics of $myoII::3xAsp$, which were identical to $myoII$ null kinetics, but they did increase the symmetry of the resulting daughter cells. Thus, daughter cell symmetry and furrow ingression kinetics appear to be established by different thresholds of myosin II activity: depletion of $rmd1$ from WT cells or RMD1 and LMM$_{TF}$ expression in $myoII::3xAsp$ cells cause the phenotype to converge to an intermediate level of myosin II function where daughter cell symmetry is normal but furrow ingression kinetics are not (Fig. 3F).

**Long lever arm partially suppresses 3xAsp**

Finally, we asked what the hierarchical relationship is between heavy chain phosphorylation regulation and myosin mechanosensing. To do this, we combined the super-mechanoresponsive myosin II motor, which has a long lever arm (2xELC), with the 3xAsp tail. 2xELC myosin II accumulates very readily in response to low applied stress, and accumulates normally to the cleavage furrow, whereas 3xAsp does neither of these (128, 131). We then examined whether a 2xELC-3xAsp myosin would behave more like 3xAsp or more WT. Indeed, the combination ($myoII::2xELC-3xAsp$) had an intermediate phenotype where more myosin accumulated at the cleavage furrow and cytokinesis resulted in greater daughter cell symmetry (Fig. 3B-C). The 2xELC-3xAsp also showed greater filament formation by TIRF (Fig. 3D). However, the 2xELC-3xAsp could not rescue suspension growth (not shown) or furrow ingression kinetics (Fig. 3E). Thus, the 2xELC could promote greater function of 3xAsp, but not to WT levels, demonstrating
that myosin II assembly is governed by both heavy chain phosphorylation and cooperative binding of the motor on actin filaments (132).

**The building of the cleavage furrow**

How myosin II localizes to the cleavage furrow cortex in dividing cells has been one of the central focuses of cytokinesis research for many years. Originally, researchers thought that myosin II accumulated to the cleavage furrow through myosin motor-actin interactions. However, the observations that headless myosin II accumulates in the furrow region, though not fully integrated into the cortex in *Dictyostelium* cells (110), and that inhibition of actin filaments with latrunculin still allowed transient cleavage furrow accumulation in *Drosophila* cells (120) indicate that other pathways help mediate cleavage furrow localization. The actin-membrane anchoring protein anillin has been proposed to provide one possible anchor (124), but structure-function analysis indicates that the putative myosin II binding site in anillin is not essential for myosin II accumulation in *C. elegans* (133). Further, an anillin homolog is not found in *Dictyostelium*, implying that if this paradigm holds that it must be carried out by other proteins in this system. Due to some functional similarities, cortexillins could be a candidate; however, myosin II still accumulates to the cleavage furrow in cortexillin I/II double mutants, albeit to a lesser extent (82).

Important regulatory pathways include the Rho-Rho kinase pathway, which phosphorylates myosin II on the regulatory light chain. Coordinately, central spindle microtubules or astral microtubules pointing towards the equator are thought to deliver
cues to the equatorial cortex (117, 134-136). These cues are likely transported in part by microtubule-based motors such as the kinesin-6 family members (e.g. MKLP1, kif12). These can then transport regulators of contractility such as Aurora kinase and INCENP in Dictyostelium and/or Rho-pathway regulators such as MgcRacGAP and ECT2 (not found in Dictyostelium, but implicated in C. elegans, Drosophila and mammalian cells) (75, 76, 78). These regulators might help direct the assembly of myosin II by activating Rho kinase (in metazoans), which promotes bipolar thick filament assembly (137-139). However, diffusion places limits on how signaling pathways alone can direct accumulation.

For a cell to build a contractile network, three fundamental questions (when, where, and which) must be addressed. The “When?” question is generally answered by signaling pathways, which often include Rho, Rho kinase, and myosin II regulatory light chain phosphorylation as well as heavy chain phosphorylation. However, from the measured cytoplasmic diffusion coefficient (~1 µm²/s; (140)) of an unassembled myosin II monomer, one can estimate that in ~10 seconds a myosin II monomer could diffuse across a small cell (~8 µm diameter) and in ~100 seconds a myosin II monomer could travel ~25 µm. Because myosin II bipolar thick filaments require approximately 100 seconds to assemble (68, 128, 132, 141), diffusive signaling alone does not readily explain where myosin II assembly will occur in small cells, such as Dictyostelium, which are 8 µm in diameter although it can help in larger cells such as mouse oocytes, which are 80 µm in diameter (142, 143). For this reason, other pathways and processes such as cortical receptors and mechanosensitive accumulation (see next) must play a
significant role in localization of myosin II. The “Where (in the cell)?” and “Which (actin filaments)?” questions are answered by myosin II-mediated mechanosensing (see next) where mechanical stresses guide where myosin II assembles on favorable actin filaments (79, 128, 132, 144, 145). Track-associated proteins provide another strategy for motors to identify preferred actin filaments. Finally, cortical receptors can help localize myosin BTFs. For example, the septin-binding protein Bni5 and the iqgap homolog Iqgap1 in budding yeast provides this type of function for myosin II accumulation to the bud neck (146).

The LMM TF, myosin TF, and 14-3-3 highlight the multi-state nature of the contractility machinery and confirmed that the selection process could identify proteins that work directly on myosin II. The myosin tail fragment encoded by the LMM TF and myosin TF constructs provides additional interactions that stabilizes 3xAsp, helping it to assemble. As discussed previously, we have shown that 14-3-3 modulates the assembly of myosin II bipolar thick filaments to control the distribution and ultimately cortical tension of the cell (122). Overexpression of 14-3-3 did not rescue the localization of 3xAsp myosin II. However, reducing 14-3-3 expression levels using 14-3-3hp, which promotes formation of larger myosin II assemblies (122), allowed 3xAsp myosin II to accumulate at the cleavage furrow. Collectively, these observations point to the fact that 3xAsp is not completely dead for assembly and localization, the system is likely to be multi-state, and the myosin tail fragment and 14-3-3 shift the populations around the multiple states. By doing so, these proteins then drive the system towards poor assembly and no localization, too much assembly and aberrant localization, or the
proper balance of assembly and localization, depending on the combination of proteins expressed. The data presented here identify an additional feature of cytokinesis; namely, that there are different thresholds of myosin II activity for ensuring daughter cell symmetry and normal furrow ingression kinetics (Fig. 3F). Expression of LMM$_{1F}$ or RMD1 in the myoII::3xAsp cells was able to restore daughter cell symmetry but not furrow ingression dynamics.

Thus, we have identified that myosin II should be considered a multi-state system, where an ideal balance of assembly and localization must be achieved for proper accumulation and cleavage furrow contractility (Scheme 1). Either too much or too little assembly leads to a poisoned system. This led us to consider other factors that the cell uses to achieve the appropriate levels of myosin assembly.
Chapter 3. Inhibiting myosin assembly

Given the importance of 14-3-3 proteins across eukaryotic species, the role of phosphorylation in the regulation of myosin-II assembly, and the demonstrated interaction between 14-3-3 and myosin-II in Dictyostelium, we examined the interplay between these proteins in detail to determine whether 14-3-3 represents a hitherto unknown regulator of cell mechanics. Through a series of in vitro studies, we uncovered a novel method by which cell mechanics are regulated in eukaryotes in which 14-3-3s act directly on the myosin tail to govern thick filament assembly.

Human 14-3-3 sigma, which is widely expressed in epithelial tissues, has been particularly well studied in the cancer literature and is often downregulated in breast, colorectal, liver, and bladder cancers (147-150). 14-3-3 sigma is both induced by and an activator of the p53 transcription factor in response to DNA damage, where it promotes the maintenance of the G2/M checkpoint. Since bypassing this checkpoint is a major promotor of genetic instability, it is no surprise that the loss of 14-3-3 sigma expression is common in tumors. However, a more inexplicable finding is that 14-3-3 sigma is often overexpressed in late-stage pancreatic, lung, and gastric cancers, where it correlates with increased metastasis and poorer clinical outcomes (151-153). How this tumor suppressor protein can become an oncogene under certain conditions, but not in others, remains largely unexplored.
14-3-3 inhibits assembly of Dictyostelium myosin-II

We have demonstrated that Dictyostelium 14-3-3 inhibits the assembly of full-length myosin-II in vivo and in vitro (93). Due to the importance of heavy chain phosphorylation in myosin-II assembly and the large number of proteins known to interact with 14-3-3 in a phospho-dependent manner, the role of phosphorylation in this inhibition is of great interest. One hypothesis is that 14-3-3 sequesters phosphorylated myosin monomers, thus reversibly removing them from the assembly pathway and hindering phosphatase access. Indeed, the Eukaryotic Linear Motif (ELM) resource (http://elm.eu.org/), which predicts protein interactions and modifications based on known motifs (most of which are phosphorylated), uncovered seven potential 14-3-3 interaction sites along the myosin-II heavy chain. Two of these were proximal to known phosphorylation sites. However, since the preparation of myosin-II from Dictyostelium involves sequential rounds of assembly and disassembly, the final product is expected to be largely unphosphorylated, as heavy chain phosphorylation is inhibitory to filament formation. Therefore, any in vitro interaction between purified myosin-II and 14-3-3 is likely to be phosphorylation independent.

To reconcile the ELM predictions with experiments and to eliminate the possibility of residual phosphorylation within the myosin prep, we examined 14-3-3’s effect on phosphomimetic as well as non-phosphorylatable Dictyostelium myosin-II tail fragments. We generated several mCherry-tagged myosin-II tail fragment constructs (Fig. 4A): ADCT (which begins at the minimal myosin-II Assembly Domain and ends at the C-Terminus of the heavy chain, residues 1531-2116), ADCT 3xAla (ADCT with the
three regulatory threonines at 1823, 1833, and 2029 mutated to alanines), ADCT 3xAsp (ADCT with the same threonines mutated to aspartic acid), and AD only (the minimal assembly domain, residues 1531-1824 (154)). The mCherry fusion allowed for the use of fluorescence techniques, and provided a globular head to promote the formation of ordered filaments as described by Hostetter et al. (155). Each construct was expressed in *E. coli*, which lacks the eukaryotic kinases to phosphorylate *Dictyostelium* myosin-II, and purified using column chromatography.

After verifying that the mCherry-tagged ADCT construct assembled appropriately, we observed that this assembly was inhibited in the presence of 14-3-3, mirroring the behavior of full-length myosin ([Fig. 4B](#)) (93). The 3xAla ADCT construct assembled to a higher degree than WT ADCT, and the 3xAsp ADCT construct assembled poorly, consistent with previous *in vitro* and *in vivo* work (62). 14-3-3 was able to inhibit assembly of all three ADCT constructs ([Figs. 4B-D](#)), indicating that the interaction is not phosphorylation-dependent.
Since phosphorylation was not necessary for 14-3-3 to solubilize myosin, we reasoned that the tail regulatory region is unlikely to be the binding site. We examined whether the minimal myosin assembly domain alone could be solubilized by 14-3-3. This mCherry-AD protein assembles in a salt-dependent manner similar to that of full-length *Dictyostelium* myosin-II, although to a lesser degree. Indeed, 14-3-3 robustly disrupts assembly of this construct, indicating that the binding site for 14-3-3 lies within the minimal myosin-II assembly domain (Fig. 4E).

**Figure 4.** In *Dictyostelium*, 14-3-3 solubilizes myosin-II by directly binding to the minimal assembly domain in a phosphorylation-independent manner

A. Schematic shows myosin-II and constructs derived from it. Highlight, minimal assembly domain. Black arrows, critical regulatory threonines or substitutions thereof. Blue and red arrows, ELM-predicted 14-3-3 interaction sites. Red barrels, mCherry.

B-E. Salt-dependent sedimentation of: B, ADCT, C, ADCT 3xAla, D, ADCT 3xAsp, E, AD only. A lower soluble fraction corresponds to more complete assembly. 14-3-3 increases the soluble fraction in all cases.

F. Titration of 14-3-3 against AD. K_d=300 nM dimer.

G. Titration of AD. Increasing amounts of AD lead to increased assembly, 14-3-3 reduces assembly. All points represent mean ± SEM.

H. Synthetic peptide competition assay with *Dictyostelium* mCherry-AD and 14-3-3. Peptides have no effect on myosin assembly or on 14-3-3 inhibition of myosin assembly.

**14-3-3 binds the minimal AD of *Dictyostelium* myosin-II**

Since phosphorylation was not necessary for 14-3-3 to solubilize myosin, we reasoned that the tail regulatory region is unlikely to be the binding site. We examined whether the minimal myosin assembly domain alone could be solubilized by 14-3-3. This mCherry-AD protein assembles in a salt-dependent manner similar to that of full-length *Dictyostelium* myosin-II, although to a lesser degree. Indeed, 14-3-3 robustly disrupts assembly of this construct, indicating that the binding site for 14-3-3 lies within the minimal myosin-II assembly domain (Fig. 4E).
The ELM resource predicts that two potential 14-3-3 interaction sites lie within the minimal assembly domain of Dictyostelium myosin-II, although this tool was trained on the primarily phosphorylated 14-3-3 interactome. Furthermore, neither of these sites have been reported to be phosphorylated in vivo. Nevertheless, we tested whether these domains were the sites of 14-3-3 binding by synthesizing non-phosphorylated REVVTID and KLESDI peptides (the two ELM-predicted binding sites in the myosin tail) and performed a competition assay. Neither of these peptides had any effect on myosin assembly nor on 14-3-3’s ability to solubilize myosin (Fig. 4H). While this could be due to structural differences between the coiled-coil myosin tail and the unstructured peptide, other unphosphorylated peptides are known to bind 14-3-3 tightly and to robustly inhibit its interaction with target proteins (156). Therefore, we concluded that the ELM-predicted sequences were unlikely to be the sites of 14-3-3 binding. Due to the sensitivity of myosin-II’s assembly activity to perturbations within this minimal assembly domain, and the lack of any well-defined rules for predicting unphosphorylated 14-3-3 binding sequences, we did not map the binding region at a higher resolution.

To determine the affinity of the interaction between 14-3-3 and myosin-II, we performed titration experiments. When 14-3-3 was titrated against a constant concentration of mCherry-AD, we obtained the expected saturation binding curve, with a $K_D$ of approximately 300 nM$_{dimer}$ (Fig. 4F). Since the assembly of myosin-II itself is also concentration-dependent (assembly will proceed until the concentration of unassembled myosin-II is equal to the critical concentration for assembly), we
performed the reverse experiment and attempted assembly of the minimal AD at different myosin concentrations, with and without 1 µM dimer 14-3-3. Indeed, the fraction of myosin in the soluble phase decreased with increasing myosin concentration, and the soluble fraction increased at each point upon the addition of 14-3-3 (Fig. 4G). Taken together, these results demonstrate that the assembly-inhibiting interaction between Dictyostelium 14-3-3 and myosin-II is direct, non-phosphorylation-dependent, and maps within the minimal assembly domain of the myosin tail.

**Mammalian myosin-II tail fragments form ordered filaments in vitro**

In higher eukaryotes, the spectrum of possible 14-3-3-myosin-II interactions is broader, as there are three paralogs of myosin-II and seven paralogs of 14-3-3 in the mammalian genome. We hypothesized that at least some of these interactions would lead to the sequestration and solubilization of myosin, similar to what was observed in Dictyostelium. To determine which interactions demonstrated this behavior, we expressed and purified 6xHis-tagged versions of all seven human paralogs of 14-3-3 (beta, epsilon, gamma, eta, theta, sigma, and zeta). We also generated and purified mCherry-fused tail fragments of each mammalian myosin-II (human IIA (MYH9), human IIB (MYH10), and mouse IIC (MYH14)) that comprised the assembly competence domain (ACD) and the non-helical tailpiece (Fig. 5A, D). Untagged tail fragments of this region have been described previously (157). The ELM bioinformatics tool predicted a conserved 14-3-3 interaction region within the assembly competence domain of each paralog.
All three non-muscle myosin-II constructs formed ordered filaments as observed by negative-stain EM (Fig. 5B). Filaments were allowed to assemble at 150 mM NaCl prior to being placed on the grid for mCherry-IIB and mCherry-IIC, whereas 25 mM NaCl was used for IIA to promote sufficient filament formation. Assembly of mCherry-IIB at a lower ionic strength resulted in the formation of larger fiber-like structures, suggestive

**Figure 5. Mammalian myosin II tail fragments form ordered filaments**

A. Schematic shows the mammalian myosin-IIs and constructs derived from them. Highlight, regions necessary for filament formation (assembly competence domain and non-helical tailpiece). Red and green arrows, ELM-predicted 14-3-3 interaction sites. Red barrels, mCherry.

B. Representative negative-stain EM images of mCherry-IIA, mCherry-IIB, and mCherry-IIC filaments. IIA was assembled at 25 mM NaCl, IIB and IIC were assembled at 150 mM NaCl. Scale bar represents 100 nm. Images were uniformly adjusted for brightness/contrast and gamma to enhance visibility.

C. EM of IIA and IIB assembled in the presence of 14-3-3 theta, showing long ribbon-like structures.

D. Coomassie blue stained gel of purified proteins used in this study. Variation in size is partly due to different affinity tag length.
of side-polar assembly of the tail fragments. These large fibers were similar in appearance to EM images obtained using an unlabeled IIB assembly domain construct (157). We measured the filaments formed by each construct and found that mCherry-IIA formed filaments of 69.7±8.6 nm, and mCherry-IIIC of 77.5±14.3 nm. The mCherry-IIB construct formed the best-ordered filaments, with a clear banding pattern and average filament length of 67.5±8.6 nm. We were unable to obtain EM images of unassembled tail fragments. This is not surprising, since unassembled tail fragments are on the order of ~120 kDa, which can be difficult to visualize with conventional uranyl acetate negative staining (158). We concluded that our tail fragments could recapitulate myosin assembly and were suitable for studying the effects of 14-3-3 on myosin.

**14-3-3 sigma reduces the size of myosin-II filaments**

To test our hypothesis that 14-3-3s could affect myosin assembly in the mammalian system, we used analytical size exclusion chromatography to determine the degree of filament assembly. We first performed assays on mCherry-IIB alone, which was pre-assembled at varying salt concentrations and then loaded on the column for analysis. As we decreased the salt concentration from 500 mM (a level which precludes assembly) to 150 mM (matching the physiological ionic strength in human cells), the partition coefficient (K_AV) of the eluted peak decreased, corresponding to the formation of larger assemblies (**Fig. 6A**). Furthermore, the shape of the peak, which at high ionic strength appeared as a single Gaussian corresponding to one species, shifted to a skewed distribution as ionic strength decreased, signifying that multiple larger species were eluting.
Figure 6. 14-3-3 sigma solubilizes myosin-II filaments

A. Analytical sizing on mCherry-IIB pre-assembled at listed salt concentrations. Decrease in eluted peak $K_{av}$ indicates filament assembly.

B. Analytical sizing on mCherry-IIB preassembled at 150 mM NaCl, alone or with 14-3-3 sigma. 14-3-3 sigma leads to a pronounced shift toward smaller filaments.

C. Same as B, but assembled at 250 mM NaCl. 14-3-3 sigma fully disassembles filaments, as indicated by superposition of the eluted peak with unassembled dimers at 500 mM NaCl.

D. FCS autocorrelation curves of mCherry-IIB assembled at listed salt concentrations. Higher peak autocorrelation values correspond to greater filament assembly.

E. FCS diffusion time for filaments assembled with or without 14-3-3 sigma. IIIB assembled at 500 mM NaCl is included as unassembled control. Diffusion times for both IIA and IIIB are markedly larger, indicating filament assembly. Both are solubilized by 14-3-3 sigma.

F. Titration of 14-3-3 sigma against mCherry-IIB. Dimers per filament are calculated as the inverse of the independently diffusing particle count, normalized against unassembled dimers at 500 mM NaCl. $K_D=330$ nM dimer. All points represent mean ± SEM.

G. FCS raw particle count for mCherry-IIA at various ionic strengths or with additional 14-3-3 sigma.

H. As above, for mCherry-IIB
Mixing 14-3-3 sigma with pre-assembled tail fragments at 150 mM NaCl prior to the run shifted the eluted peak to a smaller size, indicating that 14-3-3 sigma disassembled myosin-II filaments (Fig. 6B). In addition, the inclusion of 14-3-3 sigma with smaller myosin-II filaments (pre-assembled at 250 mM NaCl) resulted in a $K_{AV}$ matching that obtained at high salt, indicating complete 14-3-3-mediated disassembly of myosin-II filaments (Fig. 6C). Thus, despite the differences between the regulation of myosin-II assembly in Dictyostelium and humans, human 14-3-3 sigma appears to solubilize at least one paralog of human myosin-II.

**Human 14-3-3 sigma perturbs myosin-II assembly in solution**

To examine myosin assembly at physiological temperatures, we employed fluorescence correlation spectroscopy (FCS). Here, the fluorescence emissions of a ~1 fL confocal volume are monitored for a given time and an autocorrelation analysis is performed on this data. This allows for measurement of the average number of independently diffusing fluorescent species within the volume, as well as their average diffusion time (159). First, we performed FCS on mCherry-IIB tail fragments at high salt, where assembly is inhibited. The measured diffusion time was 406±10 µs, which is in good agreement with the theoretical value of 388 µs based on the measured Stokes’ radius from our analytical gel filtration experiments.

We monitored the number of independent particles and their diffusion time as the ionic strength was lowered. As myosin-II filaments assemble, the number of independently diffusing fluorescent species will drop. This causes an increase in the peak autocorrelation value, which is indeed what we observed (Fig. 6D). The diffusion
times for both myosin-IIA and IIB were higher (indicating slower diffusion) at the physiological ionic strength than at high salt, and furthermore, the addition of 14-3-3 sigma at the physiological ionic strength reduced the diffusion time of both significantly (Fig. 6E). These changes in diffusion time match our analytical sizing results and demonstrate that 14-3-3 sigma reduces the size of myosin-II filaments. However, due to the rod-like nature of the filaments and the unknown diffusional changes occurring upon 14-3-3 binding, the relationship between filament size and diffusion is complex and difficult to model. Therefore, we focused our analysis on the independent particle count, which provides a direct readout of filament size if the same total myosin concentration is used in each case.

We determined that the mCherry-IIB tail fragment formed filaments of 10-12 coiled-coil dimers on average at 150 mM NaCl, which is approximately half the value estimated from EM image analysis of full-length myosin-II (160, 161). Adding 14-3-3 sigma yields a reduction in the average filament size in a dose-dependent manner, indicating that 14-3-3 inhibits assembly of filaments in solution (Fig. 6F, H). Similar data were obtained for mCherry-IIA (Fig. 6G). Fitting a simple hyperbolic binding model to this data yielded an apparent $K_D$ of 330 nM$_{dimer}$, which is strikingly similar to the $K_D$ obtained for the Dictyostelium 14-3-3-myosin-II interaction.

14-3-3’s effect on myosin-II assembly is 14-3-3-paralog-specific

To determine whether all seven 14-3-3 paralogs disassemble myosin-II, we used sedimentation assays to track the effects of each 14-3-3 on myosin-II assembly. We performed a salt-dependent assembly assay on mCherry-IIB and observed that adding
an equimolar amount of 14-3-3 sigma resulted in robust mCherry-IIB solubilization, with
the greatest effect occurring at 150 mM NaCl. Surprisingly, the use of 14-3-3 theta
sharply contrasted with this behavior and drove mCherry-IIB further into the assembled
state (Fig. 7A). To determine whether one of these effects would dominate in the same
tube, we performed a competitive cross-titration of both 14-3-3s at various molar ratios
against a constant amount of IIB. We observed that the level of mCherry-IIB assembly
was directly proportional to the ratio of 14-3-3s, with more theta leading to greater
assembly and more sigma leading to disassembly (Fig. 7B). An equimolar mix of theta
and sigma led to baseline IIB assembly. To compare our sedimentation data to the FCS

Figure 7. Human 14-3-3s alter myosin-II assembly in a paralog-specific fashion
A. Salt-dependent sedimentation of mCherry-IIB, alone or in the presence of 14-3-3 sigma or theta. A
lower soluble fraction corresponds to more complete assembly.
B. mCherry-IIB assembled at 150 mM NaCl in the presence of 14-3-3 sigma and theta, at various molar
ratios.
C. Titration of 14-3-3 sigma against IIB at 100 mM NaCl. K_D=270 nM_dimer.
D-F. Sedimentation assay of D, mCherry-IIA, E, mCherry-IIB, F, mCherry-IIC, assembled at 150 mM NaCl,
alone or in the presence of each 14-3-3 paralog (beta, epsilon, gamma, eta, theta, sigma, zeta). Numbers
on columns are experimental replicates. All points represent mean ± SEM.
results, we titrated 14-3-3 sigma against a constant 1 µM$_{dimer}$ of mCherry-IIIB (Fig. 7C). Fitting a simple binding model to this data yielded a $K_D$ of 270 nM, in good agreement with the value obtained by FCS.

We expanded our sedimentation studies to all three myosin-II paralogs, both alone and in the presence of each 14-3-3 paralog, capturing the entire landscape of 14-3-3’s regulation of myosin-II assembly. While five 14-3-3s solubilized myosin-II, two paralogs (zeta and theta) promoted myosin assembly (Fig. 7D-F). Even more surprisingly, the amount of 14-3-3 detected in the assembled fraction did not vary between solubilizers and over-assemblers, suggesting that 14-3-3 did not bind to fully assembled filaments. While the solubilization effect is likely the result of 14-3-3-mediated sequestration of myosin-II dimers, biasing the overall myosin-II population towards a less assembled state, this does not explain how 14-3-3s theta and zeta could cause overassembly. To determine if structural differences between ordinary myosin-II filaments and those formed in the presence of over-assemblers could account for the overassembly effect, we performed EM on mCherry-myosin-IIA or IIB assembled in the presence of 14-3-3 theta. We found that both IIA and IIB generated large fibers (Fig. 5C). We were unable to obtain EM images of myosin filaments formed in the presence of 14-3-3 sigma, likely due to the insensitivity of the technique to unassembled or smaller filaments. Using FCS, we observed a marked decrease in the overall fluorescence upon adding 14-3-3 theta to a mCherry-IIIB solution. A confocal section of the system revealed that the addition of 14-3-3 theta had caused the formation of large
Figure 8. SPR traces for 14-3-3 binding to immobilized unassembled myosins
A. Human mCherry-IIA immobilized to sensor chip. Various 14-3-3s are introduced sequentially and demonstrate specific binding to myosin (rising traces), followed by wash-out with buffer and slow release of binding (falling traces).
B. As above, using mCherry-IIB
C. As above, using mCherry-IIC
myosin ribbon-like structures of mCherry-IIIB, matching our EM observations but making FCS measurements impossible.

**14-3-3 binds unassembled myosin-II specifically**

Because our readout for 14-3-3 binding in the previous data was its effect on myosin filament size, we wished to obtain a direct readout of binding independent of assembly. To do this, we employed surface plasmon resonance (SPR). In this method, a “bait” analyte is immobilized on the surface of a sample chip. Light reflected off of the bottom of this chip excites an electromagnetic surface wave, or plasmon, whose propagation properties are highly sensitive to surface conditions. Upon the addition of liquid-phase “prey”, any binding to the “bait” will alter surface conditions enough to produce a detectable signal. Since SPR requires no labeling of the species being analyzed and is performed with immobilized bait, we felt that it would be ideal for studying the binding of 14-3-3 to unassembled myosin monomers under physiological conditions. We immobilized these monomers to monoclonal antibodies in a high-salt buffer to prevent assembly. Once immobilized, we switched to a physiological ionic strength, and tested each 14-3-3 for binding. We were able to observe specific binding in every case except for 14-3-3 zeta (Fig. 8). 14-3-3 zeta appears to be the least stable isoform, as I have previously noted that its ability to affect myosin assembly (by sedimentation) diminishes well before any others when liquid aliquots are kept on ice. Therefore, we cannot draw any conclusions from its failure to demonstrate binding by SPR. The SPR technique also provides an estimate of \( K_D \), but due to the extreme
divergence from reality that binding unassembled, immobilized myosin tail fragments represents, we did not draw any conclusions from this data.

**Mutagenesis of key 14-3-3 residues alters myosin-II assembly**

Since 14-3-3 proteins are highly conserved, we performed a sequence alignment on all seven paralogs to determine whether any features differed between assembler and solubilizer 14-3-3s (Fig. 9A). We noted that 14-3-3s theta and zeta shared residues at positions 25, 34, and 91 that were not shared by the other paralogs, and hypothesized that these residues could prove important for the overassembly effect. Examining the crystal structure of 14-3-3 theta revealed that all three residues were located on the back side of the protein, opposite the canonical binding pocket (Fig. 9B). To test the sufficiency of these residues for conferring the myosin assembly character of a 14-3-3, we generated single amino acid substitution mutants of 14-3-3s theta and sigma, our strongest over-assembler and solubilizer respectively. Sedimentation on mCherry-IIIB assembled in the presence of each mutant revealed that 14-3-3 sigma was sensitive to certain mutations, but continued to solubilize myosin-II in all cases except for E34A (Fig. 9C). However, mutating any of these residues in 14-3-3 theta led to a complete phenotypic conversion from over-assembly to solubilization (Fig. 9D). Particularly interesting is the fact that K49E, a charge-substitution mutant known to disrupt interactions with many 14-3-3 targets, nonetheless solubilizes myosin-II in both 14-3-3 paralogs.
Figure 9. Altering critical residues in 14-3-3 converts an assembler to a solubilizer

A. Sequence alignment of all seven 14-3-3 paralogs. Only the first half of the alignment is shown. Red arrows—residues shared by overassembler 14-3-3s theta and zeta, but not by other paralogs.

B. Crystal structure of 14-3-3 theta, with key residues highlighted. Blue & Green, subunits of the 14-3-3 dimer. Yellow, K49, the key lysine in the canonical binding pocket contacting phosphorylated ligands. Red, the three residues highlighted in A, not positioned near the canonical binding pocket.

C. Sedimentation assay on mCherry-IIB at 150 mM NaCl, alone or in the presence of 14-3-3 sigma point mutants. Certain mutants of 14-3-3 sigma weaken its solubilization activity.

D. Same as C, but with 14-3-3 theta point mutants. All point mutants of 14-3-3 theta convert it from an overassembler of myosin to a solubilizer.

E. Sedimentation assay on mCherry-IIB at 150 mM NaCl showing the effects of the 14-3-3 sigma inhibitor BV02. The DMSO control recapitulates 14-3-3 sigma and theta as a solubilizer or overassembler of myosin, respectively. Inclusion of BV02 has no effect on baseline IIB assembly or 14-3-3 theta, but disrupts 14-3-3 sigma-mediated myosin solubilization. All points represent mean ± SEM.

F. Analytical sizing runs on 14-3-3s alone. Note preceding peaks for 14-3-3 theta, implying different multimerization states. Difference in large peak is due to differences in construct size.

G. Analytical sizing runs on 14-3-3 theta mutants. Presence of preceding peaks has no correlation with overassembler phenotype.
To determine whether our 14-3-3 preparations had similar behaviors in the absence of myosin-II, we performed analytical gel filtration on 14-3-3s sigma and theta, as well as the 14-3-3 theta substitution mutants. Although all 14-3-3s are very similar in size and shape, we observed that while 14-3-3 sigma eluted as a single Gaussian species, 14-3-3 theta had a succession of larger MW peaks in the eluate, suggesting that it is capable of forming higher-level oligomers (Fig. 9F). However, the 14-3-3 theta mutants (which converted to solubilizers) also demonstrated these preceding peaks, signifying that oligomerization does not confer overassembler activity (Fig. 9G).

To examine this further, we obtained a small molecule inhibitor of 14-3-3, BV02. BV02 is a competitive inhibitor of 14-3-3 sigma, and has been shown to drive apoptosis in cell culture by disrupting the interaction between 14-3-3 sigma and c-Abl (162). Mixture of BV02 with 14-3-3 sigma completely disrupted its ability to solubilize mCherry-IIB by sedimentation, but had no effect on 14-3-3 theta (Fig. 9E). No published literature exists on whether BV02 is capable of inhibiting other 14-3-3s besides sigma, although the binding pocket is well conserved. Therefore, 14-3-3 theta may interact with myosin-II through a different interface or in a different conformation within its binding pocket. Alternatively, BV02 may not bind 14-3-3 theta. Overall, these results paint a compelling picture of 14-3-3 paralogs competing to govern the degree of myosin-II assembly within the cell, providing a modulation of myosin activity which is independent of canonical myosin heavy and light chain phosphorylation.
Chapter 4. Conclusions

We have demonstrated that 14-3-3 acts as a buffering system for myosin-II motor proteins, directly interacting with the C-terminal tail domain to promote myosin-II assembly or disassembly. S100A4 proteins use a related mechanism to inhibit myosin-IIA assembly (163, 164). However, all three paralogs of mammalian myosin-II are modulated by 14-3-3s, and their ability to inhibit or drive assembly provides a more diverse repertoire of outcomes for myosin-II assembly. We demonstrated that in Dictyostelium, 14-3-3 is able to solubilize both phosphomimetic and non-phosphorylatable myosin-II tail fragments, and in mammals, 14-3-3s can solubilize unphosphorylated tail fragments. Therefore, 14-3-3 modulation of myosin-II assembly does not require phosphorylation of the myosin tail. The overall level of myosin-II assembly is likely achieved by a combination of canonical phospho-regulation and 14-3-3 binding. Since myosin-II phosphorylation and dephosphorylation is an enzymatic step, whereas 14-3-3 binding is non-enzymatic, phosphorylation could serve to govern the timing and gross magnitude of myosin-II assembly, while 14-3-3 would modulate myosin-II assembly continuously to ensure the availability of assembly-competent myosin-II monomers around the cell.

This regulatory paradigm becomes particularly interesting in the context of cells which express multiple 14-3-3 paralogs. Since different 14-3-3s have varying effects on myosin assembly, 14-3-3 paralogs likely compete for myosin-II within a single cell. The relative expression levels of each paralog would, therefore, alter the average level of myosin-II assembly. Furthermore, myosin-II must compete with other 14-3-3 interactors
for occupancy. Since the majority of known 14-3-3 interactors are phosphorylated, these binding events may be of higher affinity than the myosin-14-3-3 interaction. Therefore, the degree of 14-3-3 contribution to myosin-II assembly becomes a function of relative protein levels. Myosin-II is highly expressed in most cells, in the 4 µM_{dimer} range in Dictyostelium (43), and likely similar levels in mammalian cells. 14-3-3 is also highly expressed in many cells, and is found at 0.7 µM_{dimer} in Dictyostelium (93). If these were the only proteins in the cell, those concentrations and our measured \( K_{D} \) of 270 nM would dictate that 92% of 14-3-3 would be bound to myosin. Other 14-3-3 targets are expressed at differing levels, and often the unphosphorylated form of these targets does not bind 14-3-3, and therefore, will not compete with myosin. Thus, changes in available 14-3-3, either through shifts in its own expression or in the availability of phosphorylated targets, could alter the levels of myosin-II assembly around the cell. This is similar to what has been observed in Dictyostelium, where altering 14-3-3 levels has dramatic effects on myosin-II assembly as well as on cortical tension (93). As myosin-II force production is a major contributor to cell mechanics in both systems, this would make the myosin-14-3-3 interaction a significant determinant of overall cell mechanics.

This concept is quite compelling in the context of 14-3-3 sigma, which has many protective roles for epithelial cells, but whose expression patterns change upon tumorigenesis (165). In breast, colon, lung, pancreatic, and kidney cancers, levels of 14-3-3 sigma expression increase. The loss of 14-3-3 sigma expression in early-stage tumor growth is easy to rationalize, as this paralog plays roles in apoptosis, cell cycle control,
and DNA damage repair. However, the field has been unable to explain why this tumor suppressor protein would be overexpressed in late-stage metastatic cancers. Our finding that 14-3-3 is a solubilizer of non-muscle myosin-II provides one possible explanation. Metastatic cancers are often mechanically softer than regular tissue or non-metastatic cancer (166, 167). These tissues are also more mechanoresponsive (168). This seeming contradiction can be reconciled by considering the increased myosin-II turnover caused by 14-3-3 sigma sequestration of myosin-II monomers. In fact, decreasing myosin-II expression has also been shown to lead to increased invasiveness and tumor formation in vivo (169). Particularly relevant to this work is the finding that increased tumor formation on myosin-IIA knockdown or knockout is complementary to p53 inactivation. While a link between myosin II and p53 has been elusive, our findings demonstrate a clear link between the two, as both are 14-3-3 interactors. Thus, in addition to the other deleterious effects that p53 inactivation carries for the cell, p53 downregulation will also lead to a greater abundance of 14-3-3 sigma in the cytoplasm. As in Dictyostelium, excess 14-3-3 sigma would be expected to promote more dynamic myosin-II assemblies with greater turnover, promoting increased mechanosensitivity and thereby the ability of myosin II to polarize, driving 3D motility (170). Thus, we have demonstrated a novel role for 14-3-3 proteins in a cell mechanics pathway that is conserved from amoebas to humans, and which may play an important role in both healthy and disease states.
Chapter 5. Future Directions

In this thesis, we have explored the factors that govern myosin assembly and mechanosensitive localization, and biochemically characterized the conserved myosin-14-3-3 pathway using purified Dictyostelium and human proteins. However, while the importance of this pathway has been clearly demonstrated in vivo for Dictyostelium, with only one 14-3-3 and one non-muscle myosin II, this has not been done in human cells, with seven 14-3-3s and three non-muscle myosin IIs. This is partly due to the challenge of detecting a phenotype arising from a potential 21 combinations of myosin and 14-3-3. Fortunately, in most cells the repertoire of protein expression will only allow a more limited number of combinations than this theoretical maximum, but the possibility of multiple partially compensatory effects remains. We have attempted to overexpress labeled versions of 14-3-3s theta and sigma in HeLa cells without any apparent effect on myosin localization or overall levels of assembly (by sedimentation); however, it is worth noting that the effects of 14-3-3 overexpression in the Dictyostelium system were very subtle. Indeed, the cytoskeletal consequences of this overexpression only truly came to light in the context of a second stressor, such as the addition of nocodazole to perturb microtubules. Thus, it is perhaps unsurprising that the more complex human interactome would show no obvious phenotype. However, the consequences of 14-3-3 knockdown in Dictyostelium were far more severe and easily characterized. Therefore, we could attempt similar experiments in human cells by depleting 14-3-3 with specific shRNAs.
Another possibility in lieu of a knockdown, with all of its attendant concerns about specificity, population variability, and compensatory regulatory changes, is acute treatment of cells with a specific 14-3-3 inhibitor. Several such inhibitors exist, including the peptide R18, a dimeric peptide containing the R18 sequence known as difopein (dimeric fourteen-three-three peptide inhibitor), or the non-peptidic 14-3-3 inhibitor BV02. R18 and difopein have the advantage that they can be expressed within cells from a DNA construct. As in Dictyostelium, complete inhibition of 14-3-3 is lethal in human cells, inducing apoptosis within hours (171). However, any effects that 14-3-3 has on cell mechanics should precede the induction of apoptosis, allowing us to capture them with an appropriate timecourse. We will note that a biosensor for 14-3-3 function in the cell exists—the phosphatase cdc25C contains a nuclear localization sequence (NLS), but is phosphorylated and retained in the cytoplasm by 14-3-3 during interphase. Dephosphorylation of cdc25C upon entry into M phase allows the protein to enter the nucleus, and mutation of this sequence causes cdc25C to assume a pancellular distribution and promotes premature chromosome condensation (172). We have generated a GFP-labeled version of cdc25C, which will provide us with a direct readout of 14-3-3 activity in the cell.

We are also intrigued by the overassembly phenomenon that we observed for certain 14-3-3-myosin interactions. One disquieting possibility is that this overassembly is simply an artifact resulting from the affinity tag remaining on our purified proteins, although why this would affect certain 14-3-3s more than others (including point mutants of the same protein!) is unclear. We have attempted to enzymatically cleave
the affinity tag and generate unlabeled 14-3-3s, but achieving specific cleavage has proven to be a challenge. Therefore, we have generated label-free constructs for bacterial expression and are in the process of purifying unlabeled, full-length 14-3-3s sigma and theta to answer this question. Another interesting study direction in Dictyostelium would be a more careful dissection of the interplay between myosin assembly and mechanosensitivity. We have shown that co-expression of 3xAsp in a background of WT myosin II carries a deleterious effect, whereas expressing 3xAsp alone while simultaneously enhancing its mechanosensitivity (3xAsp with 2xELC in cis) can effect a partial rescue of WT behavior. Is this due to changes in filament size, mechanoresponsiveness, or both? Would co-expression of the more mechanosensitive 2xELC in trans with 3xAsp also partially rescue WT behavior? Would the two myosins co-assemble into hetero-BTFs? How would the strength of this “mechanosensitivity rescue” compare to directly rescuing assembly by expression of tail fragment “assembly templates”? What of cells under additional mechanical stress (for example, from agarose overlay? Furthermore, knockdown of 14-3-3 also promoted the assembly of 3xAsp and reduced its deleterious effects on cell growth. Can overassembly (3xAla) be similarly rescued by overexpression of 14-3-3? If 3xAsp and 3xAla are co-expressed in the appropriate ratio to produce a cell with normal overall levels of myosin assembly, but no means to regulate this assembly, what are the consequences for cytokinesis? Answering these questions would shed much light onto the complex regulatory factors that govern cell division, as well as myosin force generation around the cell.
Chapter 6. Materials and Methods

Reagents and protein purification

Bacterial expression plasmids coding for an N-terminal 6xHis tag, fused to the mCherry fluorophore, fused to the assembly domains of Dictyostelium myosin-II (residues 1533-1823), human myosin-IIA (residues 1722-1960), human myosin-IIB (residues 1729-1976), and mouse myosin-IIC (residues 1782-2033) were generated in pBiEx1 using standard cloning techniques. Bacterial expression plasmids for 6xHis-tagged Dictyostelium 14-3-3, human 14-3-3 epsilon, human 14-3-3 sigma, and human 14-3-3 zeta were generated in pBiEx1 as well. Bacterial expression plasmids for human 14-3-3s beta (Addgene plasmid # 39128), gamma (Addgene plasmid # 39129), eta (Addgene plasmid # 38814), and theta (Addgene plasmid # 38931) were gifts from Nicola Burgess-Brown. Proteins were expressed in BL-21 Star™ (DE3) (Invitrogen) E. coli in LB shaking culture overnight at room temperature. Bacteria were harvested by centrifugation and lysed by lysozyme treatment followed by sonication, and the lysate was clarified by centrifugation. Polyethyleneimine (PEI) was added to a final concentration of 0.1% to precipitate nucleic acids, which were then removed by centrifugation. 14-3-3 precipitated in the PEI pellet for Dictyostelium 14-3-3 and human 14-3-3 epsilon. This pellet was therefore resuspended in column running buffer (10 mM HEPES, pH 7.1, 500 mM NaCl, 10 mM imidazole) and dialyzed against the same for a minimum of 4 hours, clarified by centrifugation and filtration, and run on a Ni-NTA metal affinity column to obtain high-purity 14-3-3. The myosin-II constructs and human 14-3-
3s beta, gamma, eta, sigma, theta, and zeta remained in the PEI supernatant and were precipitated by adding ammonium sulfate to 50% saturation and centrifuging. The pellet was resuspended in column running buffer, dialyzed against the same for a minimum of 4 hours, clarified by centrifugation and filtration, and run on a Ni-NTA metal affinity column, followed by a sizing column for the myosin-II constructs. The myosin-II constructs were then concentrated and further purified by dialyzing against assembly buffer (10 mM HEPES, pH 7.1, 50 mM NaCl) until precipitate formed, followed by centrifugation and resuspension of the pellet in storage buffer (10 mM HEPES, pH 7.1, 500 mM NaCl). Protein purity was verified by SDS-PAGE followed by Coomassie Blue staining, and concentration was quantified by UV absorbance using the calculated extinction coefficient for each protein’s amino acid sequence.

The 14-3-3 inhibitor, BV02, was purchased from Sigma (Sigma SML0140). REVVTID and KLESDI peptides were synthesized by the Synthesis & Sequencing Facility at the Johns Hopkins University School of Medicine using an Aapptec Focus Synthesizer.

**Analytical gel filtration**

Analytical gel filtration was performed using a BioLogic DuoFlow FPLC system (Bio-Rad) and a Superdex 200 10/300 GL column (GE). After equilibration with running buffer (10 mM HEPES, pH 7.1, 500 mM NaCl), the void volume and total bed volume were obtained with blue dextran in water. The column was then calibrated using sizing standards of aprotinin, cytochrome c, carbonic anhydrase, BSA, and thyroglobulin. The volume of elution was measured from the beginning of sample injection to the peak of the UV trace, and a $K_{av}$ for each species was obtained using Equation 1. A calibration
plot of $R_H$ vs. $K_{AV}$ was generated and fitted. Experimental runs were performed in the same fashion, allowing for the calculation of $R_H$ for each unassembled mCherry-myosin-II paralog. Assembly was monitored by re-equilibrating the column at a lower salt concentration (250 or 150 mM NaCl), diluting a sample to the same concentration, and monitoring the shift in the UV peak. All runs were performed at 0.5 ml/min flow rate, 0.5 ml total injection volume, and 4 °C.

$$K_{AV} = \frac{v_F - v_0}{v_t - v_0}$$

**Assembly assay**

*In vitro* assembly of myosin-II was conducted according to the method of Zhou *et al* (122), with a number of modifications. The protein concentration for each species in the tube was increased to 1 µM$_{dimer}$ to ensure that the smaller protein was adequately visible by Coomassie Blue staining, and the incubation time and temperature were adjusted to 30 minutes at the physiological temperature for each myosin species (22 °C for *Dictyostelium* myosin, 37 °C for human myosins). These temperatures were also used during the centrifugation step. Band intensities post-staining were quantified and background subtracted using ImageJ (NIH). The integrated density of the supernatant, divided by the summed integrated densities of the supernatant and the pellet, yielded the soluble fractions for each sample. $K_D$ was determined by fitting **Equation 2** to a titration experiment using a least-squares approach.

$$\frac{X-X_{min}}{X_{max}-X_{min}} = \frac{[L]}{[L]+K_D}$$
**Fluorescence correlation spectroscopy**

All FCS measurements were performed using a Zeiss LSM780-FCS confocal microscope with a Zeiss C-Apochromat 40x/1.2 water immersion objective and a stage warmer to maintain the sample at 37 °C. The confocal volume was calibrated using a 50 nM solution of rhodamine-6G in water, with a diffusion coefficient of 400 µm²/s at 22.5 °C (175), which equates to a diffusion coefficient of 572.3 µm²/s at 37 °C (Equation 3).

\[
D = \frac{k_B T}{6\pi \eta R_H}
\]

To measure myosin filament assembly, mCherry-labeled myosin tail fragments were diluted in a solution containing 10 mM HEPES, pH 7.1, at a desired concentration of NaCl, and the temperature was allowed to equilibrate to 37 °C for at least 10 minutes. The final concentration of tail fragments was either 100 nM$_{\text{dimer}}$ or 50 nM$_{\text{dimer}}$, and the salt concentrations used were 500 mM NaCl (for completely disassembled filaments), 250 mM NaCl (for partial assembly), or 150 mM NaCl (for assembly at a physiological ionic strength). Other proteins (14-3-3 sigma, 14-3-3 theta, purified BSA at 0.5 µg/ml) were included as needed. Since the concentration of the fluorescent species is a crucial parameter to normalization, we verified that raw counts remained the same across conditions, indicating that non-specific aggregation or adherence to the walls of the chamber was not a factor. We also observed no effects on raw counts or the independent particle count when BSA was added to the solution as a nonfluorescent blocking protein.
After equilibration, FCS measurements were taken as 10 consecutive 5-second scans. Any scans that demonstrated aggregates, baseline drift, or other aberrant features were discarded, and an autocorrelation curve was generated for the remaining scans and fitted with a 1-component, 1-triplet state model using ZEN imaging software (Zeiss). Particle counts and diffusion times were extracted from these fits, and a minimum of 10 points per sample were averaged to generate the final measured values. We confirmed that the diffusion coefficient for mCherry-IIB at high salt matched well with our analytical sizing results and therefore was likely to be diffusing as the fully unassembled dimer, and used the particle count under this condition to normalize the other mCherry-IIB particle counts. We then reported the number of mCherry-IIB dimers per filament under each experimental condition as the inverse of this normalized particle count. $K_D$ was determined by fitting Equation 2 to a titration experiment using a least-squares approach.

Electron microscopy

Carbon-coated EM grids were rendered hydrophilic by glow discharge. Freshly prepared grids were placed in sample droplets for 30 seconds, followed by 2 washes in water and staining using 1% uranyl acetate. Samples were then observed using a Philips BioTwin CM120 Transmission Electron Microscope.

Dictyostelium Strains, Constructs, and Cell Culture

*Dictyostelium discoideum* strains used in this study are wild type Ax3 (Rep orf+ cells) (2) and *myoll heavy chain* null mutant (*mhcA* (HS1)) (173). Cells were cultured in
Hans’ enriched HL-5 media (1.4x HL-5, containing 8% FM, 60 U/ml penicillin and 60 µg/ml streptomycin sulfate) and selected with drugs for plasmid transformations with 15-30 µg/ml G418, 4 µg/ml blasticidin, and/or 10-60 µg/ml hygromycin as appropriate. Cells were propagated at 22°C on 10-cm Petri dish plates. For suspension growth, cells were cultured in 10-ml culture volumes in 125-ml Erlenmeyer flasks at 180 rpm, 22°C. Cell densities were determined by counting cells on a hemocytometer.

Myosin II was observed by using pBIG:GFP-myosin II or pDRH:mCherry-myosin II (122, 174) and pBIG:GFP-3xAsp or pDRH:GFP-3xAsp constructs (126). For the cDNA library selection, WT::3xAsp cells with stable integration of GFP3xAsp were generated by linearizing the plasmid pDRH:GFP-3xAsp by restriction enzyme digestion and then transforming into wild type (Ax3(Rep orf+)) cells. To identify stably integrated clones, sub-clones of cells were subjected to suspension culture to select pools that have constant IC-50 growth reduction even in the medium without antibiotic selection; GFP-3xAsp expression was monitored throughout suspension to make sure that the fluorescence intensity remained at its original level even after many cycles of suspension culture. We then performed western analysis of WT and WT::3xAsp cells to measure the fraction of myosin II that was endogenous wild type myosin II (60%) and GFP-3xAsp myosin II (40%). These cells were then used for cDNA library suppression.

The myosin tail fragment (nucleotides 5671-6351) was amplified using polymerase chain reaction (PCR) and ligated into pDM181. 2xELC-3xAsp was engineered by combining myosin 2xELC and 3xAsp cDNAs. The rmd1hp was constructed in the pLD1A15SN (pLD1) vector, using PCR to amplify nucleotides 188-690 with Sal I at the 3’
end and Not I at the 5’ end. When sub-cloned into the Sal I and Not I sites of pLD1, the fragment was in the antisense orientation, yielding pLD1:rmd1AS (690-188). Nucleotides 388-690 were amplified with Not I and Mlu I sites introduced at the 5’ and 3’ ends, respectively. Upon sub-cloning into the Not I and Mlu I sites in pLD1:rmd1AS, the resulting plasmid encoded pLD1:rmd1hp with the stem composed of 388-690 and the loop composed of 388-188.

**Light microscopy**

Cells were imaged using an Olympus 1X81 microscope with a 40x (NA 1.3; for epifluorescence imaging) or a 60x (NA 1.49; for total internal reflection fluorescence imaging) objective with 1.6x optovar. Images were acquired using the MetaMorph Software (Molecular Devices). For live cell fluorescence imaging, cells were plated on imaging chambers for at least 30 min. and then the media was replaced with MES buffer (50mM MES [pH 6.8], 2mM MgCl₂, 0.2mM CaCl₂) right before imaging to reduce auto-fluorescence. Unless otherwise noted, cells were imaged with DIC (10-ms exposure) and GFP (40-ms exposure).

**Cytokinesis Analysis**

Log phase cells were screened under the microscope and both DIC and GFP images were collected for the dividing cells. Images were processed with ImageJ software (http://imagej.nih.gov/ij/). The furrow concentration of GFP-3xAsp was quantified using the ratio of background subtracted mean fluorescence intensity at the cleavage furrow (Iᵢ) and the polar cortex (Iᵢₒ). The intensity ratio of Iᵢ/Iᵢₒ was calculated
and used for statistical analysis. Furrow ingression dynamics were measured as described previously (7). Relative diameter was determined by normalizing the measured diameter by the crossover distance $D_x$ and the time axis was shifted so that the time-point in which $D_x$ was reached was reset to 0. $D_x$ is the point in which the furrow length and diameter were equal.

To determine daughter cell symmetry, we measured the 2D-cross-sectional area and calculated the ratio of the large cell to the small cell as described previously (122). The data sets were analyzed by Kruskal-Wallis and Wilcoxon nonparametric tests using KaleidaGraph (Synergy Software).
**Contributions**

I would like to acknowledge Yixin Ren, Jon Osborne, Christopher Miller, Alexandra Surcel, and Douglas N. Robinson for their contributions to this study. Yixin Ren was responsible for the initial 3xAsp suppressor screen, as well as imaging and analysis of the RMD1, RMD1hp, actin, LMM\textsubscript{TF}, and 14-3-3hp lines. Christopher Miller helped with the TF cloning and suspension growth recapitulation. Jon Osborne performed the *Dictyostelium* ADCT, ADCT 3xAsp, and ADCT 3xAla purification and sedimentation. Robert Bloch and Yinghua Zhang at the University of Maryland Biosensor Core performed the SPR studies.

Part of this work was published in and modified from “Genetic suppression of a phosphomimic myosin II identifies system-level factors that promote myosin II cleavage furrow accumulation” (130) and “Cytokinesis mechanics and mechanosensing” (176). Both journals give authors the right to include their articles in full or in part in a thesis or dissertation, provided that this is not to be published commercially.
Bibliography


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Curriculum Vitae

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EDUCATION

Expected graduation date: January 2016

2009 – 2016  Johns Hopkins School of Medicine, Baltimore, MD  
PhD, Cell Biology  
Thesis advisor: Douglas Robinson  
Thesis project: Regulation of non-muscle myosin II

2002 – 2005  University of Washington, Seattle, WA  
BS, Chemical Engineering  
Minor in Chemistry  
GPA: 3.47  
Performed composites research (Two quarters)  
Conducted proton-exchange membrane research (One quarter)

2000 – 2002  Bethel College, Mishawaka, IN  
Pre-Engineering  
GPA: 3.75  
Dean’s List (All four semesters)

PROFESSIONAL EXPERIENCE

2008 – 2009  Research Technician (Immunology)  
Seattle Children’s Research Institute, Seattle, WA
• Performed directed evolution of yeast surface-displayed homing endonucleases via successive rounds of mutagenesis and FACS
• Developed assays to characterize homing endonuclease cleavage specificity and efficiency

2006 – 2008  Research Technician (Geophysics)
Department of Earth and Space Sciences, University of Washington, Seattle, WA

• Designed, machined, assembled, and operated equipment for high-pressure viscosity experiments
• Prepared samples and performed measurement of mineral crystal elastic constants via impulse stimulated scattering
• Calibrated, programmed, and maintained laser optics-based instrumentation

2002 – 2005  College Technician (Information Technology)
Rainier Beach High School, Seattle, WA

• Managed Active Directory network
• Performed troubleshooting and repair of software, hardware, peripherals, and network issues school-wide
• Installed new hardware and software for teachers and administrators

RESEARCH EXPERIENCE

Publications

West-Foyle H, Osborne J, Robinson DN. 14-3-3 proteins tune non-muscle myosin II assembly, providing a bridge between cell mechanics and cancer metastasis. In preparation.


**Oral presentations**

**2015**

Non-muscle myosin-II assembly is tuned by 14-3-3 proteins. Cell Biology departmental seminar, Johns Hopkins University, Baltimore, MD.

**2014**

14-3-3 proteins tune non-muscle myosin-II assembly, providing a possible bridge between cell mechanics and cancer metastasis. ePoster Talk, presented at the 2014 ASCB/IFCB Annual Meeting, Philadelphia, PA.

14-3-3 proteins tune non-muscle myosin-II assembly, providing a possible bridge between cell mechanics and cancer metastasis. Presented at the
Biochemistry, Cellular and Molecular Biology Annual Retreat, St. Michaels, MD.

Myosin assembly regulation by 14-3-3. Cell Biology departmental seminar, Johns Hopkins University, Baltimore, MD.

2013
Myosin assembly regulation by 14-3-3. Biochemistry, Cellular and Molecular Biology Colloquium, Baltimore, MD.

Myosin assembly regulation by 14-3-3. Cell Biology departmental seminar, Johns Hopkins University, Baltimore, MD.

Force Fluctuations within Focal Adhesions Mediate ECM-Rigidity Sensing to Guide Directed Cell Migration. Cell Biology departmental seminar, Johns Hopkins University, Baltimore, MD.

2012
The dynamics of myosin regulation. Cell Biology departmental seminar, Johns Hopkins University, Baltimore, MD.

The dynamics of myosin regulation. Biochemistry, Cellular and Molecular Biology Colloquium, Baltimore, MD.

2011
RNA Mimics of Green Fluorescent Protein Cell Biology departmental seminar, Johns Hopkins University, Baltimore, MD.

Poster presentations


West-Foyle H, Osborne J & Robinson DN. (2014) 14-3-3 proteins tune non-muscle myosin-II assembly, providing a possible bridge between cell


Abstracts


TEACHING EXPERIENCE

2011 – 2014 Summer Academic Research Experience (SARE) Mentor

Served as a personal mentor to three high school students over four summers performing bench research, providing technical training, and teaching group and one-on-one academic lessons in biology, chemistry, and physics, as well as professional skills development.

AWARDS

2014 Lewis Travel Award Honorable Mention
2013 American Society for Cell Biology Graduate Travel Award Recipient of the Isaac Morris Hay and Lucille Elizabeth Hay Graduate Fellowship
2012 1st Place winner, Biochemistry, Cellular, and Molecular Biology (BCMB) recruitment poster session
2011 1st Place winner, BCMB recruitment poster session
2009 Thomas J. Kelly, MD, PhD and Mary L. Kelly Young Scholar Fund