

14-3-3, an integrator of cell mechanics and cytokinesis

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One of the goals of understanding cytokinesis is to uncover the molecular regulation of the cellular mechanical properties that drive cell shape change. Such regulatory pathways are likely to be used at multiple stages of a cell's life, but are highly featured during cell division. Recently, we demonstrated that 14-3-3 (encoded by a single gene in the social amoeba *Dictyostelium discoideum*) serves to integrate key cytoskeletal components—microtubules, Rac and myosin II—to control cell mechanics and cytokinesis. As 14-3-3 proteins are frequently altered in a variety of human tumors, we extend these observations to suggest possible additional roles for how 14-3-3 proteins may contribute to tumorigenesis.

the major suppressors identified was overexpression of 14-3-3.⁴ 14-3-3 proteins are acidic ~30 kDa proteins, which dimerize to form a protein-binding cleft that often, but not always, binds phosphorylated target proteins.⁵⁻⁹ 14-3-3 proteins are known to interact with several hundred proteins involved in a wide array of cellular processes such as apoptosis, cell cycle progression and cytokinesis so their involvement in cell division is not unanticipated.^{6,10,11}

14-3-3 and RacE

In *Dictyostelium*, 14-3-3 is enriched in the cell cortex in a manner dependent on the RacE small GTPase.⁴ A portion of 14-3-3 also fractionates into an insoluble pool that depends on RacE and GTP, suggesting that active RacE is essential for the cortical binding. 14-3-3 overexpression was able to rescue the severe cortical mechanical and cytokinesis defects of *racE* null mutants, solidly implicating a RacE-14-3-3 pathway. The RacE isoform in *Dictyostelium* is a Rac-family small GTPase that is a little more distantly related to canonical Rac members of mammals and is found to be somewhat related to Rac and Rho subfamilies.¹² *Dictyostelium* contain several typical Rac isoforms, which interact with p21-binding domains (PBDs) found in proteins such as pak proteins (p21-activated kinases).¹³ RacE does not interact with PBDs,¹⁴ and it is unclear what the relevant direct downstream effectors of RacE are. However, the dependence of 14-3-3 on RacE for cortical distribution and its solubility along with the ability of 14-3-3 overexpression to rescue several *racE* phenotypes indicates that 14-3-3 interacts closely with and downstream of RacE,

Introduction

Cytokinesis is the process by which a mother cell divides into two daughters cells. Inherently mechanical, this process depends on the integration of the entire cytoskeletal network as multiple processes must be coordinated in space and time.¹ Traditionally, the mitotic spindle, the network of microtubules that separates the chromosomes, has been considered the primary source of the regulatory cues that coordinate events at the cell cortex.² While trimeric G-proteins and regulators of Rho small GTPases have been implicated in a number of systems,^{2,3} several holes and in some cases inconsistencies exist in our understanding of how the mitotic spindle regulates the cortical remodeling that drives cytokinesis. To begin to address this, we used a chemical genetic approach to identify suppressors of nocodazole (a microtubule depolymerizer), and one of

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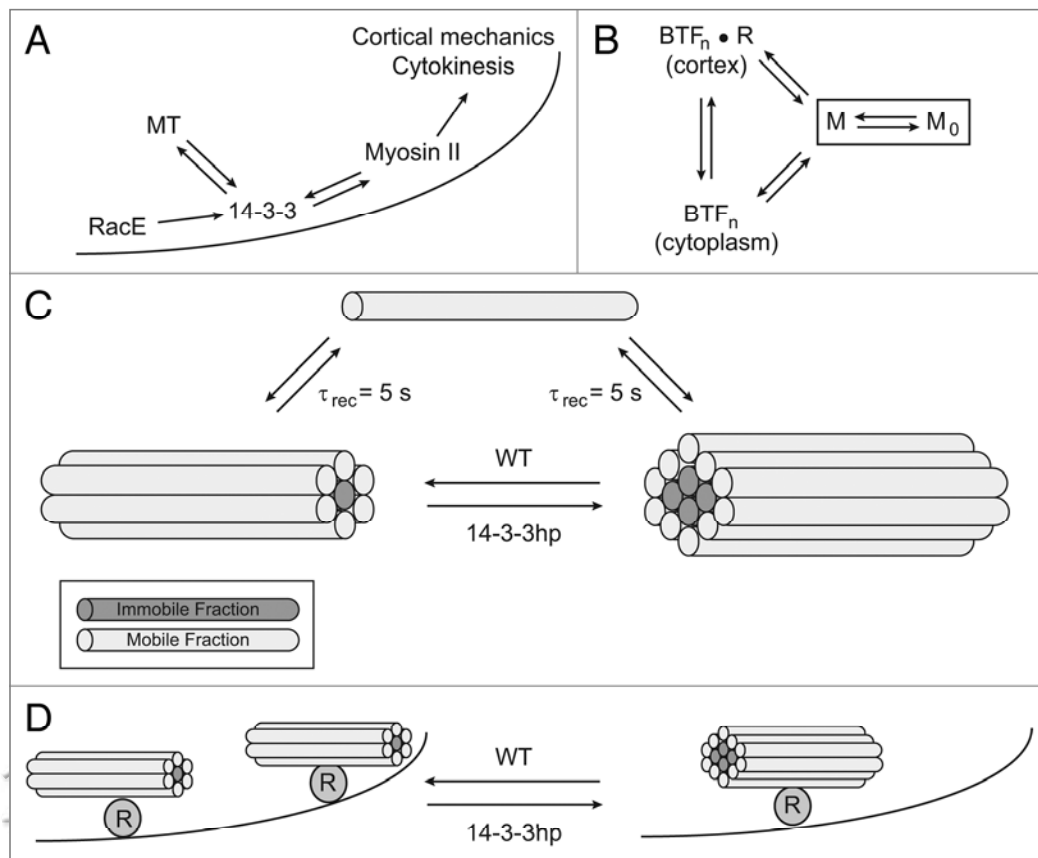


Figure 1. 14-3-3 coordinates RacE, microtubules and myosin II. (A) A pathway diagram demonstrating how 14-3-3 operates downstream of RacE and microtubules (MT) but is required to maintain MT length. 14-3-3 also promotes myosin II bipolar thick filament (BTF) remodeling along the cortex (depicted by the curved line) in order to modulate cortical mechanics and cytokinesis shape change. (B) Myosin II BTF assembly occurs through a nucleation-elongation process.⁴³ The assembly incompetent form of myosin II (M_0) is maintained by heavy chain phosphorylation. The assembly competent form (M) can nucleate BTFs with n monomers per BTF. These can be formed in the cytoplasm or on the cortex. Some evidence suggests that cortex anchoring may occur through some receptor R that binds to the coiled coils of the BTFs.^{44,45} (C) Alterations in 14-3-3 expression levels do not affect the recovery time ($\tau_{rec} = 5$ s) measured by FRAP. However, the immobile fraction is increased by 14-3-3 depletion using a 14-3-3 RNAi hairpin vector (14-3-3 hp). One way to achieve this is if the myosin monomers M (depicted as rods with motor domains not shown) are able to release with similar kinetics, but if 14-3-3 helps maintain smaller BTFs (which are predicted to be exponentially distributed by size⁴⁶), then fewer monomers will be buried deep within the BTF (BTFs assemble through lateral interactions so that the BTF primarily grows by increasing in diameter rather than length). (D) 14-3-3 (along with RacE) might also form part of a cortical receptor complex (R). If the cortical receptors also help nucleate the BTFs, fewer receptors (for example by depleting 14-3-3 with 14-3-3 hp) would lead to fewer nucleation centers. This might then increase the immobile fraction as depicted in (C).

though a direct interaction has not yet been detected (Fig. 1A).

14-3-3 and Myosin II

Though most organisms have two or more 14-3-3 isoforms (mammals have seven), the Dictyostelium genome has just one. It is not surprising then that 14-3-3 is an essential gene in this system.⁴ However, partial silencing of 14-3-3 to 30% of normal levels using a hairpin plasmid (14-3-3 hp) led to cell cultures that produced multinucleated cells, a hallmark of cytokinesis failure. The dynamics of cleavage furrow contractility and the morphology of the 14-3-3 hp cells as their shaped evolved was

highly reminiscent of the very stereotypical *myosin II* null cytokinesis. Subsequent proteomics analysis revealed that myosin II heavy chain is a major binding partner for 14-3-3. Follow-up analysis confirmed that 14-3-3 does indeed bind wild type myosin II though not the phosphomimic 3x Asp mutant myosin II, which is myosin II bipolar thick filament (BTF) assembly incompetent.

Three well characterized consensus binding motifs are commonly found in 14-3-3-binding partners.⁹ These include two phosphorylation-dependent binding motifs (motif 1 and motif 2) and a non-phosphorylation-dependent motif (motif 3). However, other types of interaction

sites have also been observed. Interestingly, Dictyostelium myosin II has no predicted consensus motif 1 binding sites, only motifs 2 and 3, most of which are found in the coiled coil tail.⁴ All of the predicted sites near the 3x Asp mutant sites are of the motif 3 class, suggesting that the 14-3-3-myosin binding interactions may not be directly dependent on phosphorylation. The inability of 14-3-3 to associate with 3x Asp myosin II may be because 3x Asp is severely impaired in its ability to assemble into bipolar thick filaments¹⁵ or its cellular distribution is shifted from the cortex to the cytoplasm.¹⁶ Alternatively, subtle structural changes in the myosin II tail upon phosphorylation might reduce

the affinity of the 14-3-3-myosin II interaction, thereby reducing the association of 14-3-3 with the phosphomimic 3x Asp myosin II.¹⁷

Nevertheless, assessment of the myosin II distribution in cells with altered 14-3-3 levels demonstrated that 14-3-3 promotes myosin II BTF remodeling (Fig. 1B): depletion of 14-3-3 led to decreased myosin II mobile fraction and clumping, whereas 14-3-3 overexpression led to an increased mobile fraction (Fig. 1C). Mobility here refers to the mobile and immobile fractions of myosin II as assessed by fluorescence recovery after photobleaching. Consistent with the RacE dependency for 14-3-3 function, myosin II also shows aggregation in *racE* null cells. In in vitro myosin II BTF assembly assays, the addition of 14-3-3 reduced the amount of assembled myosin II. The combination of in vivo and in vitro data suggests that 14-3-3 might reduce the affinity or increase accessibility of the myosin II monomers (the functional monomer is the hexamer of two heavy chains, two essential light chains and two regulatory light chains) for the BTF, which in vivo leads to alterations in myosin II mobility that correlates with 14-3-3 levels.

The direct biochemical interactions between myosin II and 14-3-3 suggest an intriguing new twist in myosin II regulation. Much of the known regulation of myosin II BTF assembly occurs through heavy chain phosphorylation and dephosphorylation.¹⁵ Clearly, this level of regulation is critical for maintaining a free pool of myosin monomers so that myosin II BTFs may be remodeled during dynamic processes such as cytokinesis and cell motility. Undoubtedly, this represents just part of the story. It remains unknown how myosin II BTF nucleation is controlled and what the cortical receptor/anchor(s) are for myosin II BTFs in most contexts (here, we define the cortex as the composite of cortical actin network and the overlying plasma membrane). Where there is evidence for a receptor, such as in the case of anillin (in metazoans), the structure-function studies raise questions about whether anillin is really an anchor or alternatively one of several anchors.¹⁷⁻¹⁹ In a recent study, $\alpha4\beta1$ integrins may also provide one possible cortical anchor for

myosin IIA in mammalian cells,²⁰ demonstrating that multiple myosin II cortical receptors likely exist. As 14-3-3 is cortically enriched, one possibility is that it forms part of one such cortical receptor for myosin II. By having many receptors, the myosin II BTFs may be nucleated at several sites, yielding smaller BTFs (Fig. 1D), which in turn may also lead to greater mobility. The recovery kinetics (τ_{rec}) largely reflect the release of myosin II monomers from the BTFs, which remain unchanged between the different levels of 14-3-3 expression ($\tau_{\text{rec}} = 5$ s for WT, 14-3-3 hp and 14-3-3-overexpressing cells) (Fig. 1C). By helping set the steady state mobility of the myosin II monomers and by maintaining a uniform distribution of myosin II BTFs, 14-3-3 contributes to cortical mechanics and cell shape changes such as in cytokinesis (Fig. 1A). Depletion of 14-3-3 leads to a significant reduction in cortical tension while its overexpression could rescue the mechanical defects associated with *racE* deletion or with nocodazole treatment. However, 14-3-3 overexpression failed to alter the cortical mechanics of *myosin II* null cells further illustrating that 14-3-3 works through myosin II.

14-3-3 and Microtubules

Since we found 14-3-3 as a suppressor of nocodazole, we also analyzed the microtubule-network structure. By mapping out and measuring the lengths of several tens of microtubules, we found that 14-3-3 helps establish the steady state average microtubule length (Fig. 1A). Because 14-3-3 was cortically enriched, we paid particular attention to the microtubule-cortex contact lengths. Upon measuring around 50,000 microtubule-cortex interaction events, we concluded that 14-3-3 does contribute to the microtubule-cortex interactions but that the nature of these interactions is complex. Both loss-of-function and overexpression of 14-3-3 led to a reduction in the length of microtubule-cortex interactions though the overall lifetime of the interactions was unchanged ($\tau = 5$ s). These observations are of added interest as previous particle-tracking studies revealed that the cell cortex has super-diffusive activities that are

myosin II-dependent.²¹ Yet, in a *myosin II* null mutant cell-line, these activities could be recovered by depleting the actin crosslinking protein dynacortin.^{21,22} These observations argue then that myosin II potentiates but does not drive these super-diffusive activities. Implicated through a process of elimination but not formally proven, microtubules were suggested to be likely drivers of the super-diffusive activities. Putting it all together, one might speculate that 14-3-3 helps anchor myosin II and microtubules at the cortex and these assemblies cooperatively modulate the super-diffusive interacting network of the cortex. One could imagine then that 14-3-3 helps link several components of the cortical myosin II-microtubule-RacE network. As an example, focal adhesions can stabilize microtubules,²³ and integrins have been recently shown to bind to myosin IIA²⁰ and 14-3-3 proteins²⁴⁻²⁶ in mammalian cells.²⁶ Thus, such a complex network could allow cross-talk from microtubules to myosin II, and 14-3-3 is poised to provide part of the critical link. Interestingly, 14-3-3 binding to $\beta2$ integrin in human T cells also leads to activation of Rac1 and Cdc42 small GTPases though how 14-3-3 leads to Rac1 and Cdc42 activation in this context is not yet fully understood.²⁴ This sort of conceptual network then could interact cooperatively to facilitate the remodeling of the cortex to promote cell shape changes and to control cortex mechanics.

14-3-3, Cell Mechanics and Cytokinesis

One of the more fascinating aspects of cytokinesis is how molecular-scale dynamics lead to cellular-scale mechanical properties that ultimately drive cell shape change.²⁷ These mechanical properties include cortical and cytoplasmic viscoelasticity, cortical tension and actively generated stresses (force/area). The list of proteins that contribute to cell mechanics include myosin II, actin polymers, actin crosslinkers, microtubules and their regulatory proteins, such as Rac small GTPases and 14-3-3.⁴ Ultimately, the mechanical parameters generated by these proteins specify the geometry (i.e., cell shape and morphology) and shape change kinetics

(e.g., rate of furrow constriction). The properties work together to lead to other cellular-scale features such as Laplace-like pressures that result from the cortical tension and the membrane curvature. All of these factors collaborate to squeeze the cytoplasm from the mid-zone, pinching the cell into two daughter cells.

Possible Implications for Cancer Biology

While *Dictyostelium* cells do not suffer from cancer (at least from our perspective), several possible medical implications are suggested by this body of work. Alterations of 14-3-3 expression levels (both up and down) are associated with a broad variety of invasive tumor states and many tumor cell-types have alterations in cell mechanics.²⁸⁻³⁰ Furthermore, it is becoming increasingly appreciated that the fidelity of cytokinesis is crucial for maintaining chromosome stability.³¹ While much effort has rightly gone into understanding the role of 14-3-3 in cell cycle control, we suggest that by regulating myosin II dynamics, 14-3-3 may play a very direct role in the myosin II contractile networks that establish the mechanical properties (cortical tension and viscoelasticity) and that drive cytokinesis contractility. Perturbations of myosin II-mediated function would promote cell transformation, tumor progression and metastasis. Indeed, myosin II heavy chain appears on the list of potential 14-3-3 σ interactors from proteomics studies,³² and 14-3-3 has been implicated in regulating myosin light chain phosphatase as well as contractile ring disassembly in mammalian cells,^{33,34} supporting these possibilities. 14-3-3 also plays other roles during mitosis and cytokinesis such as in the regulation of the centralspindlin complex,³⁵ providing yet another means by which it may impact cytokinesis fidelity. An intriguing recent study suggests that increasing myosin II-mediated contractility enhances cytokinesis fidelity and chromosomal stability, whereas inhibiting myosin II contractility reduces cytokinesis fidelity, promoting chromosomal instability.³⁶ Thus, many of the pieces for how cytokinesis fidelity, chromosomal instability, myosin II contractility and human cancer genes work

together to promote normal and pathological cell division have been observed.

In the context of cell mechanics, one mammalian cell study has attempted to directly test the impact of overexpressing 14-3-3 ζ on cell stiffness and cell transforming potential of mammary epithelial cells (MEC).³⁷ Overexpression of 14-3-3 ζ failed to have a major impact on the mechanics of these cells. This may be analogous to our observations that 14-3-3 overexpression only measurably changed the mechanics of *Dictyostelium* cells if the cells were challenged in some way (*racE* deletion or nocodazole treatment). From our studies,^{27,38} we find that some proteins show saturable effects on mechanics so that overexpression fails to have an impact unless the system has been perturbed in some other way, while depletion leads to significant softening of the cell (as in the case of 14-3-3). In contrast, other proteins like some actin crosslinkers impact mechanics, showing an increase or reduction in stiffness and tension with overexpression or depletion, respectively. We do not yet fully understand the basis for this dichotomy. However, it would be interesting to determine whether reducing the expression levels of 14-3-3 isoforms, such as 14-3-3 σ (which is downregulated in many tumor types, suggesting potential tumor suppressor activity^{28,38,39}), affects cell mechanics.

Critical Questions

Clearly, discerning the nature of the cortical receptor/anchors for myosin II is essential to determining how myosin II and 14-3-3 interact. Pressing questions include: do multiple myosin II cortical anchors exist, what are they, how do Rac and 14-3-3 regulate or modulate the binding to myosin II, and how do these complexes interact with microtubules. In this review, a discussion of integrins has been included as one possible scenario. However, other types of membrane proteins may be just as likely to play the role of cortex anchor. Chloride channels have been shown to bind to 14-3-3 proteins in *Arabidopsis* and are found in the cleavage furrow region in mammalian cells.^{40,41,44} Further, myosin II can co-purify with a subset of chloride channel containing

vesicles.⁴² Perhaps a similar network of a chloride channel-family protein and 14-3-3 is involved in myosin II cleavage furrow anchoring. Overall, 14-3-3 mediates some amount of cross-talk between these critical elements of microtubules, myosin II and RacE small GTPase. Additionally, such cross-talk creates the foundation for feedback, allowing the integration of mechanical and biochemical pathways and processes.²⁷ What will be interesting to decipher ultimately is how the cross-talk in this system leads to cooperativity and robustness to ensure cytokinesis fidelity to protect against the genomic instability that comes from cytokinesis failure.

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