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SPOTLIGHT

Moonwalking molecular machines: Unraveling the choreography of myosin filament assembly

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We have made tremendous progress in identifying the machines that shape the architecture of actin filaments. However, we know less about the mechanisms mediating myosin assembly at the supramolecular level. In this issue, Quintanilla et al. (https://doi.org/10.1083/jcb.202305023) provide important new insights into this process.

The same molecular machines that power the athleticism of a gymnast or football striker drive the remarkable cell shape changes and cell migration events during development and homeostasis (1). Myosin motor proteins walking on actin filaments rearrange the cytoskeleton, and via cytoskeletal linkage to cell–cell and cell–matrix junctions, alter cell shape. Tremendous progress has been made in identifying machines shaping the architecture of actin filaments, modulating growth, branching, bundling, and assembly into diverse supramolecular arrays at different places and in different cell types. However, we know less about mechanisms mediating myosin assembly at the supramolecular level. Quintanilla et al. (2) provide important new insights into this process.

Cytoplasmic myosin is a large, multisubunit protein (3). The heavy chain includes a globular motor domain associated with essential and regulatory light chains, and a long α-helical tail (Fig. 1 A). Two myosins dimerize when the tails form a coiled coil—this is the myosin "monomer." In the canonical view, monomers exist in a folded, inactive conformation. This is opened when regulatory light chains are phosphorylated by kinases including Rho-regulated ROCK and calcium-regulated myosin light chain kinase (MLCK). This allows assembly of ∼30 myosin monomers by tail interactions into the classic two-headed myosin "filament."

However, actin and myosin then must assemble into the diverse supramolecular arrays seen in different cell types, ranging from stress fibers in migrating fibroblasts to the actomyosin arrays at cell junctions in endothelial cells. Unraveling the architecture of both actin filaments and the associated myosin is critical for translating differential structure into differential function. Prescient work in the 1990s provided our first insights. Scientists in the Borisy lab examined myosin organization in migrating fibroblast lamella. Pushing the limits of microscopy, they obtained remarkable images of myosin filaments stacked on one another in arrays involving dozens of filaments (4, 5). New tools recently allowed scientists to follow up this work. Combining structured illumination microscopy with fluorescent protein–labeled myosin heads, they resolved individual myosin filaments as they assembled into myosin stacks or partitioned into myosin clusters in the lamella of living migrating cells (Fig. 1 B) (6–8). Aligned myosin stacks formed two-dimensional arrays tightly apposed to the plasma membrane, where they presumably either organized or were organized by stress fibers. Myosin stacks also were seen in cytokinetic furrows of sea urchin embryo cells (9) and highly ordered, sarcomeric myosin stacks were observed in epithelial cell junctions in cultured MDCK cells (10, 11) and in vivo in inner sulcus cells of the inner ear (12).

Despite these advances, it remained unclear how myosin assembly is spatially and temporally controlled within the cell and how nascent filaments assemble into higher-order networks that facilitate contractility. Quintanilla et al. (2) first examined mechanisms regulating the spatiotemporal appearance of nascent myosin filaments. To visualize myosin, they utilized mouse fibroblasts with endogenous myosin tagged at the N-terminus with a HaloTag. They then examined whether ROCK or MLCK stimulated nascent myosin assembly, using biosensors to monitor calcium and RhoA activity (upstream regulators of MLCK and ROCK activity, respectively). The authors did not observe correlation between either signaling cascade and myosin filament appearance. Furthermore, treatment with MLCK or ROCK1 inhibitors did not decrease filament appearance, suggesting neither signaling cascade is key.

However, live imaging revealed that leading edge retractions preceded nearly all nascent myosin filament assembly. The authors thus tested whether leading edge retraction and actin dynamics directly enhance myosin filament formation. Treating cells with chemical inhibitors of actin polymerization and depolymerization, thereby stalling leading edge dynamics,

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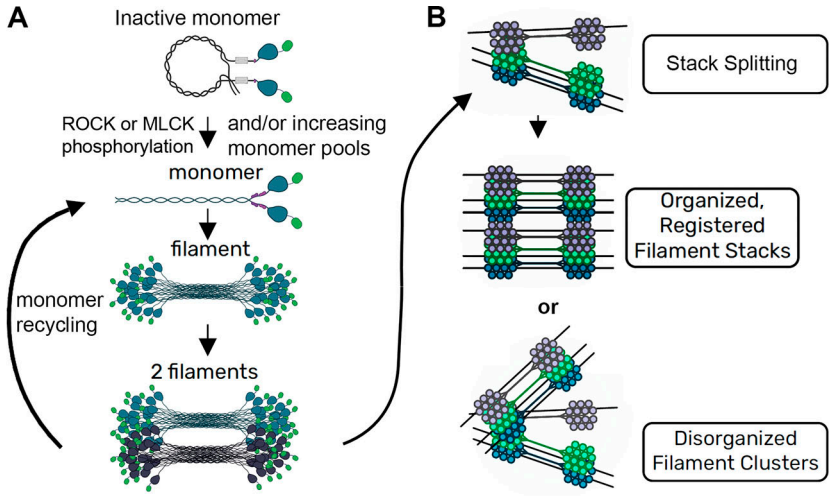

Figure 1. **Supramolecular myosin assembly. (A)** Myosin monomers begin in a closed, autoinhibited state. Phosphorylation by ROCK or MLCK relieves inhibition and allows assembly of many monomers into a two-headed myosin filament, which can further assemble into stacks. Filament disassembly can then release monomers to both feed myosin kinases and elevate cytosolic monomer levels. **(B)** The authors speculate that myosin filament "splitting" can lead to filaments remaining in register to build sarcomeric filament stacks or moving apart to generate disordered filament clusters. Images modified from Quintanilla et al. (2).

significantly decreased the rate of filament appearance, thus demonstrating that leading edge retraction and dynamic actin stimulate myosin filament assembly. Earlier work revealed that inhibiting ROCK destabilized posterior actin structures while simultaneously enhancing lamellar myosin filaments. Thus, the authors hypothesized that filament assembly is regulated by the overall cytoplasmic availability of myosin monomers. To increase cytoplasmic myosin monomer levels in a background of reduced actin dynamics, they treated cells with inhibitors stalling actin polymerization/depolymerization in conjunction with ROCK inhibitors. This dramatically elevated myosin filament assembly, supporting the idea that globally increasing cytoplasmic myosin monomer levels is sufficient to drive this.

They then used cool tools to locally increase myosin monomer levels, asking if this is sufficient to induce filament assembly. The authors employed an impressive optogenetic approach that allowed them to recruit a tagged version of myosin to the cortex of fibroblasts via blue light activation. This enabled them to use local light stimulation to spatially control recruitment of exogenous myosin and measure whether this was sufficient to initiate filament assembly. Strikingly, within minutes, locally increasing myosin monomer concentration was sufficient to promote formation of nascent myosin filaments in the absence of any changes in kinase signaling.

Next, they asked a key question in supramolecular assembly: how does a nascent myosin filament choose between initiating a new filament cluster or associating with preexisting clusters? The authors devised a novel strategy to count the number of myosin monomers present within cell lamella. They used molecular "standard candles" comprised of self-assembling 60-subunit membrane-anchored protein nanocages. Using subunits with EGFP fused to one or both termini, they created standards with 60 or 120 EGFP molecules. A third standard had a single EGFP fused to actin to identify individual fluorophores. This generated a standard curve of mean fluorescence intensity versus number of EGFPs. They then imaged endogenous EGFP-myosin in fibroblasts from knock-in mice, where every myosin monomer contains two EGFPs. Previous work revealed that mature filaments consist of ~30 monomers. Using this setup, they quantified three parameters: (1) the number of nascent myosin filament assembly events initiating new clusters, (2) fluorescent intensity increases of individual clusters within the same region during the same time, and (3) fluorescent intensity increase of all existing clusters. This allowed them to directly compare the number of nascent filament assembly events (new clusters) to the number of filaments assembling into existing clusters. Strikingly, myosin filaments exhibit an almost 100-fold higher tendency to join existing clusters rather than form new ones. Finally, the authors found that filament partitioning events involve a mature filament recruiting additional monomers to establish multiple filaments before splitting rather than a mature filament dividing into two immature filaments (Fig. 1 B).

Quintanilla et al. (2) provide important insights into the spatial and temporal regulation of myosin filament assembly. They highlight the critical role of actin and myosin dynamics in elevating monomer concentration to feed nascent filament assembly, reminiscent of the complex interplay between actin filament assembly and disassembly (13). It will be exciting to learn more about local tuning of myosin filament disassembly and monomer recycling. The authors propose a model in which filament amplification is predominately mediated by myosin:myosin interactions, whereas filament stack versus cluster formation is propelled by myosin:actin dynamics. We still do not understand the mechanisms by which filaments assemble in stacks—are myosin head interactions sufficient or do other proteins link and align filaments? Moving forward, it will be important to delve deeper into the latter part of this model to better define factors driving stack versus cluster formation. For example, while the authors associate cluster formation with disordered actin and stack formation with parallel actin movement, during cell–cell junction assembly in MDCK and Caco-2 cells, organized myosin stacks are juxtaposed with disordered actin filaments (11). In these cells, ROCK inhibition reduced cortical myosin and disrupted myosin stack organization. Thus the rules may differ in different cell types. Finally, the remarkable capacity to tackle intricate questions at a supramolecular level by combining advanced biological tools with cutting-edge microscopy techniques offers promise in many other areas.

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