Developmental Cell
Review

And the Dead Shall Rise: Actin and Myosin Return to the Spindle

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DOI 10.1016/j.devcel.2011.07.018

The spindle directs chromosome partitioning in eukaryotes and, for the last three decades, has been considered primarily a structure based on microtubules, microtubule motors, and other microtubule binding proteins. However, a surprisingly large body of both old and new studies suggests roles for actin filaments (F-actin) and myosins (F-actin-based motor proteins) in spindle assembly and function. Here we review these data and conclude that in several cases the evidence for the participation of F-actin and myosins in spindle function is very strong, and in the situations where it is less strong, there is nevertheless enough evidence to warrant further investigation.

Introduction

Spindles, the agents of chromosome segregation in eukaryotic cells, have long fascinated biologists, based on their necessity for accurate transmission of the genetic material, on their intricate beauty, and on the mysterious and dynamic manner in which they form, change, and accomplish chromosome separation. The textbook view of the spindle is of a structure based predominantly, if not exclusively, on microtubules, microtubule motor proteins such as kinesins and cytoplasmic dyneins, and other microtubule-binding proteins (Alberts et al., 2007). Indeed, most of the spindle architecture is described in terms of specific microtubule populations, structures that nucleate microtubules, and structures that attach to microtubules (Figure 1). For example, in mitotic animal cells, the spindle poles, which contain centrosomes, nucleate three populations of microtubules: the kinetochore microtubules, which extend toward the spindle midplane and attach to the condensed chromosomes at the kinetochores; the polar microtubules, which extend toward the spindle midplane and overlap polar microtubules extending from the opposite pole; and astral microtubules, which extend from the poles away from the midplane and to the cortex.

Each of these populations of microtubules is well established to participate in spindle function (Gatlin and Bloom, 2010; Goshima and Scholey, 2010; Maresca and Salmon, 2010). (Note that we use the term ''spindle function'' to refer to chromosome partitioning in general, rather than using it to refer to anaphase only, since proper chromosome segregation cannot occur in the absence of, say, proper chromosome congression or following spindle destabilization.) During spindle assembly, spindle positioning and separation of the nascent spindle poles are accomplished by cortical dynein-based pulling on astral microtubules and kinesin-based sliding of polar microtubules. Simultaneously, motors and microtubule binding proteins at kinetochores provide microtubule attachment sites on the condensing chromosomes. A complex process based on differential assembly and disassembly of microtubules at the kinetochores and poles combined with motor-based pushing and pulling jockeys the chromosomes into position at the metaphase plate. Then, by the combined activity of kinetochore motors and microtubule binding proteins that signal to the cell-cycle regulatory machinery, the cell judges whether spindle assembly is complete and, if so, executes anaphase. Anaphase, in turn, again depends on the combined activity of proteins that regulate microtubule dynamics and attachment at the kinetochores and poles and kinesin-based sliding of polar microtubules and dynein-based pulling of astral microtubules.

Given the extensive evidence in support of this general scheme, which includes an enormous number of studies in many different model systems using a variety of approaches, there would seem little reason to posit involvement of F-actin or myosins in spindle function, other than their well-known involvement in cytokinesis. However, studies going back to the 1970s, as well as much more recent work, implicate F-actin and myosins in spindles in a variety of cell types. Below, we consider this evidence broken down based on the subcellular pool of F-actin and myosins in question: ''cortical'' (directly linked to or immediately beneath the plasma membrane); ''subcortical/cytoplasmic'' (extending linearly from the plasma membrane into the cytoplasm or completely contained within the cytoplasm); or ''spindle'' (completely within the spindle and/or associated with one of the basic spindle structures referred to above). While these distinctions are useful when conceptualizing how they contribute or may contribute to spindle function, it should be noted that considerable overlap may exist in the arrangement and roles of these different pools, as in the case of F-actin cables in budding and fission yeast, which arise at the cortex but extend into the cytoplasm.

Cortical F-Actin and Myosin

Anchoring of Astral Microtubules

Spindles assume a characteristic position and orientation within cells, in X, Y, and Z, before undergoing anaphase, and this position is thought to be governed at least in part by interaction of astral microtubules with cortical anchoring or motor proteins.

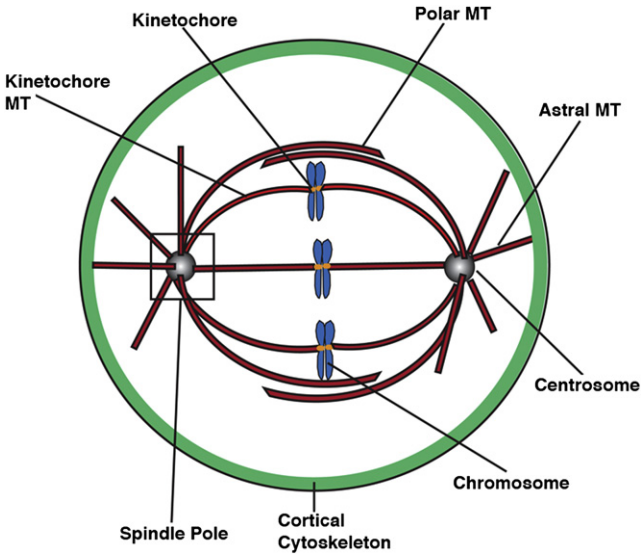

Figure 1. Schematic Diagram Depicting a Mitotic Spindle from an Animal Cell
Cortical F-actin and myosin are shown in green, microtubules (MTs) in red, the chromosomes in blue, and the kinetochores in orange.

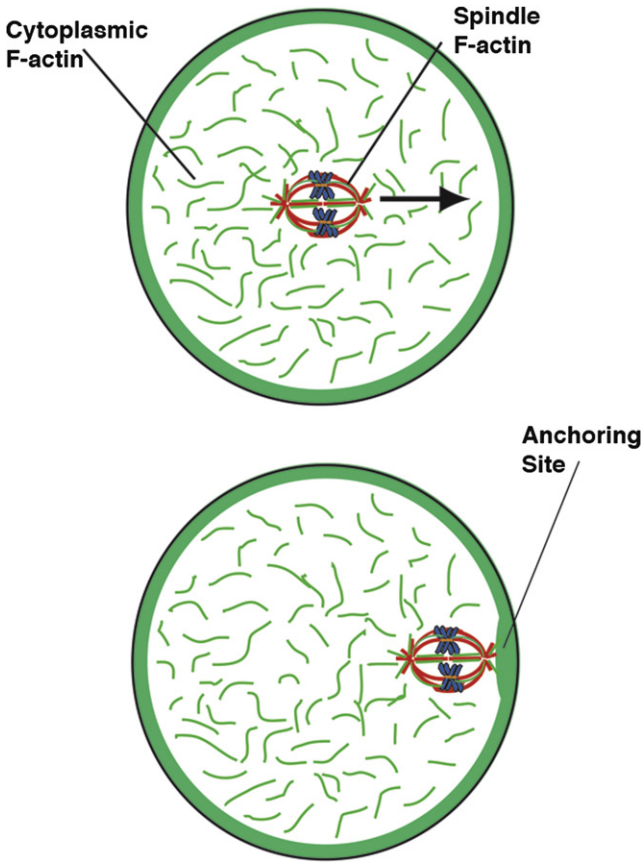

Figure 2. Schematic Diagram Depicting a Mouse Oocyte with Microtubules in Red and F-Actin in Green
Initially, the spindle, which has extensive associated F-actin, must move from the cell interior to the cortex, which it does with the help of the cytoplasmic network of F-actin cables. Once at the cortex, the spindle is anchored to a region of the cortex that is enriched in F-actin. Cortical, cytoplasmic, and spindle F-actin are shown in green, microtubules (MTs) in red, the chromosomes in blue, and the kinetochores in orange.

In many cell types, for example, spindle position is controlled by interaction of cortically localized dynein with astral microtubules (reviewed by Moore and Cooper, 2010). Whether F-actin is involved in this process is unclear (for example, see Heil-Chapdelaine et al., 2000). Nevertheless, F-actin is required for spindle anchoring and orientation in several cultured mammalian cell types, as revealed by spindle mispositioning following treatment with drugs that disassemble F-actin and the observation that this treatment results in increased spindle rotation (Théry et al., 2005; Toyoshima and Nishida, 2007). That it is cortical F-actin rather than some other pool was suggested by the fact that disruption of cell adhesions or pharmacological elimination of astral microtubules also prevented proper anchoring and orientation. It was also found that myosin-10, an unconventional myosin that binds to microtubules (Weber et al., 2004; Hirano et al., 2011), was required for spindle orientation (Toyoshima and Nishida, 2007). Because myosin-10 localizes to cell-surface projections in cultured mammalian cells (Berg and Cheney, 2002), these results are consistent with myosin-10 serving as a cortical anchor for astral microtubules.

Cortical F-actin is also involved in meiotic spindle anchoring in oocytes of several species. In these cells, a small (relative to the cell volume) spindle forms in the cell interior, migrates to the cortex (see below), and then reorients, assuming a perpendicular orientation to the cortex (Figure 2). While in some organisms spindle rotation is F-actin independent (Fabritius et al., 2011), in both frogs (Gard et al., 1995) and mice (Maro et al., 1984) it is dependent on F-actin, and, because F-actin is typically concentrated in the cortex at the site of meiotic spindle attachment (e.g., Maro et al., 1984), it is likely that, again, cortical F-actin is the relevant pool. The accumulation of F-actin apparently reflects reciprocal interactions between the spindle and the cortex in that if spindle access to the cortex is prevented, actin accumulation is perturbed. Spindle rotation in frog oocytes is also dependent on myosin-10, which localizes to both the meiotic spindle itself and the cortical region overlying the spindle, but as disruption of myosin-10 function also perturbs spindle assembly it is not clear whether the role played by myosin-10 in this process is direct (Weber et al., 2004).

F-Actin-Based Transport Functions

In addition to the apparently passive provision of astral microtubule anchoring sites, cortical F-actin and myosins can influence spindle formation or positioning in more active and, in some cases, unexpected ways. In cultured mammalian and *Drosophila* cells that fail to fully separate their centrosomes before nuclear envelope breakdown is complete, cortical F-actin and myosin-2 can finish the job by pulling astral microtubules (and thus the centrosomes) away from each other via cortical flow (Rosenblatt et al., 2004; Figure 3). Similarly, F-actin-dependent clustering of supernumerary centrosomes during mitotic spindle pole assembly was observed in *Drosophila* S2 and cancer cell lines (Kwon et al., 2008). However, whether this clustering is based on the kind of cortical flow described in Rosenblatt et al. (2004) is unknown and it may instead reflect forces exerted through

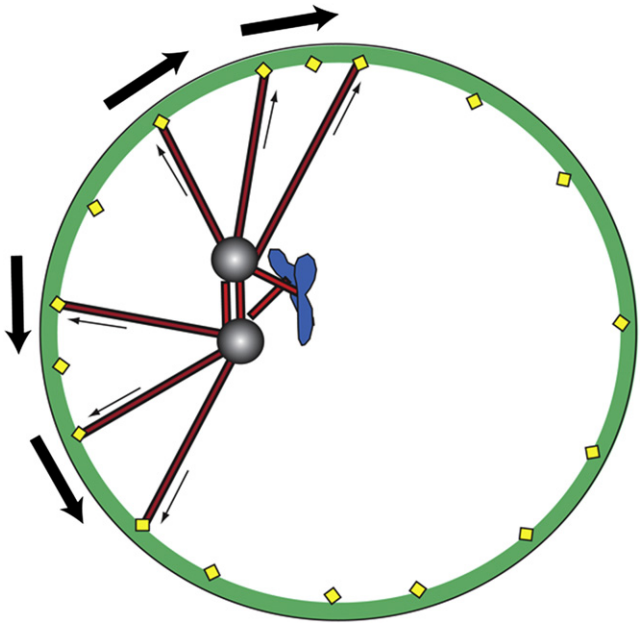

Figure 3. Schematic Diagram Depicting Separation of Nascent Spindle Poles during Mitotic Spindle Assembly Powered by Myosin-2 and F-Actin-Based Cortical Flow
The microtubules (red) are attached to the cortical network of F-actin and myosin-2 (green) by an as-yet-unidentified anchor (yellow) and to the condensing DNA (blue). Flow (arrows) moves in the plane of the cortex away from the site of closest centrosome apposition pulling the microtubules and thus the centrosomes away from each other. Gray spheres: centrosomes.

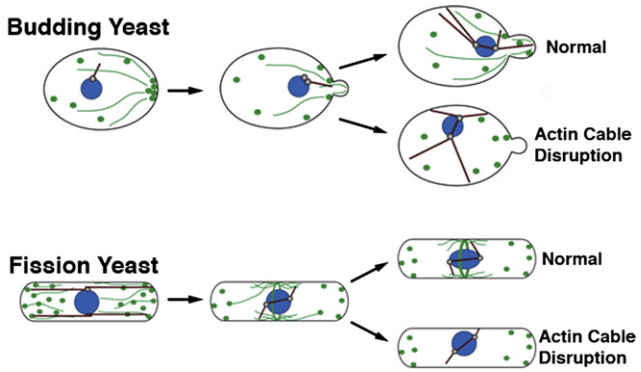

Figure 4. Schematic Diagram Depicting Interaction of Microtubules with the Spindle Pole Bodies and F-Actin Cables that Extend from the Cortex into the Cytoplasm in Budding Yeast and Fission Yeast
The consequences of specific disruption of the F-actin cables for each species is also shown. The green discs represent cortical F-actin patches and the green lines are F-actin cables. Red: microtubules; blue discs: nuclei; gray spheres: spindle pole bodies.

retraction fibers or other cortical, F-actin-based structures (Kwon et al., 2008). Myosin-6 controls mitotic spindle rotation in *Drosophila* neuroblasts, and while the means by which this occurs are uncertain, myosin-6 undergoes a redistribution during spindle rotation, suggestive of an active role (Petritsch et al., 2003). Dachs, an atypical myosin, controls spindle orientation in developing *Drosophila* wing epithelia by a completely different mechanism—that is, it drives cell shape changes, apparently via contraction, which, in turn, direct the orientation of the spindle (Mao et al., 2011).

Myosin-powered cortical flow is not the only means by which cortical F-actin controls spindle assembly. In syncytial *Drosophila* embryos centrosome separation during spindle assembly can be driven by F-actin dynamics independently of myosin-2-powered contraction (Cao et al., 2010), although a combination of cortical myosin-2-powered contraction and actin dynamics may control the final spindle length in this system (Sommi et al., 2011). Further, the rigidity that cortical F-actin and myosins impart to the cortex is also important for spindle assembly and function in some cells types (Kunda et al., 2008). Suppression of the expression or function of moesin, a plasma membrane-F-actin crosslinking protein, results in a variety of striking phenotypes including increased spindle length, abnormal chromosome partitioning, and detachment of centrosomes from the rest of the spindle (Kunda et al., 2008; Carreno et al., 2008). Likewise, a variety of spindle defects are associated with disruption of the cortical F-actin and myosin cytoskeleton in intact epithelia (Luxenburg et al., 2011). That the cause of these abnormalities is something different than simply loss of cortical flow, the basis for the centrosome separation defect, is suggested by the differences in the phenotypes themselves and by the fact that while externally applied lectins, which block cortical flow but produce extrinsic cortical rigidity, mimic the effects of myosin-2 inhibition on centrosome separation (Rosenblatt et al., 2004), they actually rescue the spindle deficits caused by moesin depletion (Kunda et al., 2008).

Cortical Signaling to the Spindle

One of the more unusual means by which cortical F-actin may control spindle function was discovered in budding yeast. Actin deploymerization in *S. cerevisiae* indirectly induces a delay prior to the onset of anaphase that is mediated by the mitotic-regulatory kinase, Swe1p (Sia et al., 1998). Depolymerization of actin stabilizes Swe1p, resulting in increased phosphorylation and inactivation of cyclin dependent protein kinase 1. However, the nature of the actin-dependent event(s) that is perturbed by actin depolymerization and sensed by Swe1p is not fully understood. Leading hypotheses propose that the cell monitors bud morphogenesis (McMillan et al., 1998) or bud size (Harvey and Kellogg, 2003), both of which are dependent on the actin cytoskeleton. Fission yeast also delay anaphase entry following actin depolymerization. Although the precise steps in the pathway linking the actin cytoskeleton to the cell cycle in *S. pombe* have yet to be delineated, the delay appears to involve the stress-activated MAPK, Sty1 (Gachet et al., 2001).

Subcortical/Cytoplasmic F-Actin and Myosins

Some of the most detailed mechanisms for spindle positioning by F-actin and myosins come from studies of yeast. Prior to anaphase, *S. cerevisiae* and *S. pombe* exhibit two prominent F-actin arrangements—cortical patches and cables. The cables, which extend from the cortex into the cell interior, are apparently the relevant species for spindle function (Figure 4). In the prevailing models, microtubules emanating from the spindle pole bodies (SPBs, nucleus-tethered structures that serve as microtubule organizing centers) interact physically with actin cables and this interaction is required for proper spindle positioning

and orientation prior to anaphase. In *S. cerevisiae* two different formins (proteins that nucleate linear actin arrays) template specific populations of actin cables (Pruyne et al., 2004) that are anchored at the cortex, either in the bud tip or near the bud neck, and extend into the cell where they interact with microtubules via a protein complex that includes Myo2p (a myosin-5), a microtubule-tip binding protein, Bim1p, and Kar9p, which links Myo2p and Bim1p (Hwang et al., 2003). Mutations in actin (Palmer et al., 1992) or the selective disruption of actin cables via mutations in formin (Lee et al., 1999) and in tropomyosin (Pruyne et al., 1998), low doses of the F-actin poison, latrunculin A (Theesfeld et al., 1999), or mutations in Myo2p (Yin et al., 2000), which specifically interacts with actin cables, cause spindle orientation defects. Similarly, disruption of actin cables in *S. pombe* results in impaired preanaphase spindle orientation and a delay in anaphase onset. Again the specific role of actin cables was indicated by the appearance of these phenotypes in formin (Gachet et al., 2004) and myosin-5 mutants (Gachet et al., 2004) or low doses of latrunculin A (Heil-Chapdelaine et al., 2000; Tournier et al., 2004).

Loss of the cytoplasmic actin cables has severe consequences for mitotic progression. Specifically, in *S. pombe* (Tournier et al., 2004), latrunculin A-treated cells exhibit a lengthened metaphase accompanied by defects in chromosome congression (Tournier et al., 2004). The observation that mutants with compromised astral microtubule-SPB interactions exhibit a lengthening of metaphase similar to that seen in latrunculin A-treated cells suggests that chromosome congression is mediated via interaction of astral microtubules with actin cables. Additionally, another study reported a metaphase-specific retardation of mitosis upon latrunculin A treatment and an associated spindle collapse (Meadows and Millar, 2008). The metaphase delay observed upon actin disruption has been suggested to involve checkpoint pathways (Gachet et al., 2001; Tournier et al., 2004), although the exact mechanism and checkpoint proteins involved are contested (Rajagopalan et al., 2004; Meadows and Millar, 2008). Experiments in *S. cerevisiae* also suggest crosstalk between the actin cytoskeleton and checkpoint pathways. For instance, in mutant yeast that lack a mitotic spindle, SPB separation is rescued by inactivation of the spindle assembly checkpoint (SAC) and this rescue is defective in the presence of latrunculin B (Chiroli et al., 2009).

Cytoplasmic F-actin cables are essential participants in the migration of the spindle from the cell interior to the cortex of mouse oocytes (Figure 2). In contrast to oocytes of several other species, wherein astral microtubule-cortex interaction promotes meiotic spindle translocation (Fabritius et al., 2011), in mouse oocytes spindle migration does not require microtubule-cortex interaction (e.g., Li et al., 2008; Schuh and Ellenberg, 2008) but is instead driven by cytoplasmic F-actin cables nucleated by formin-2 (Azoury et al., 2008; Schuh and Ellenberg, 2008) and spire, an F-actin nucleator that promotes formation of linear actin arrays (Pfender et al., 2011). The cables, detected by phalloidin staining of fixed cells (Schuh and Ellenberg, 2008) and by expression of GFP-Utr-CH (a fluorescent F-actin binding protein; Burkel et al., 2007) in living oocytes (Azoury et al., 2008; Schuh and Ellenberg, 2008), form in a meiosis regulated manner that is controlled in part by differential formin-2 expression (Azoury et al., 2011). Loss of cables via formin-2 or spire ablation has no apparent effect on cortical actin but nevertheless prevents spindle translocation. Exactly how the cytoplasmic actin cables promote spindle translocation is debated. Schuh and Ellenberg (2008) reported that the translocation is sensitive to myosin-2 inhibition (see also Simerly et al., 1998). Based on these results, and on analysis of F-actin cable dynamics, it was proposed that the spindle is pulled toward the cortex via myosin-2-powered contraction (Schuh and Ellenberg, 2008). In contrast, Li et al. (2008), using Lifeact, a fluorescent F-actin binding peptide (Riedl et al., 2008) as a probe, failed to detect the cytoplasmic F-actin cables reported by Azoury et al., (2008) and Schuh and Ellenberg (2008), but instead noted dynamic F-actin loosely associated with the spindle chromosomes. Based on this result, and on their failure to find a myosin dependence of spindle translocation, Li et al. (2008) proposed a model wherein the spindle is translocated via actin assembly-dependent pushing. The reason for the differences in the effects in myosin inhibitors is not clear, particularly as both Li et al. (2008) and Schuh and Ellenberg (2008) used externally applied ML7 at similar concentrations; however, it is worth noting that this agent was more effective at blocking polar body emission in the Schuh and Ellenberg (2008) than the Li et al. (2008) study. It should also be noted that the effects seen by Schuh and Ellenberg with ML7 were similar to those obtained in an earlier study wherein myosin-2 antibodies were microinjected (Simerly et al., 1998). With respect to the differences seen in F-actin distribution by Azoury et al. (2008) and Schuh and Ellenberg (2008) on the one hand, and Li et al. (2008) on the other, it is more than likely that this reflects the different approaches used for F-actin imaging (see below).

In addition to meiotic spindle translocation in mouse oocytes, a particularly surprising role for cytoplasmic F-actin was recently revealed by studies of starfish oocytes (Lénárt et al., 2005)—that is, chromosome congression, long thought to be the exclusive province of spindle microtubules, was shown to be driven by a closing network of F-actin that forms in association with breakdown of the nuclear (germinal vesicle) envelope (Lénárt et al., 2005; Mori et al., 2011). This network traps the condensing chromosomes as it shrinks and brings them within range for capture by microtubules of the nascent spindles. Remarkably, the great majority of chromosome movement in this system is completely independent of microtubules. It is also apparent that chromosome congression during meiosis I in mouse oocytes has no microtubule requirement (e.g., Van Blerkom and Bell, 1986), although whether this reflects a collapsing meshwork of F-actin as found in starfish oocytes or the mechanisms discussed above for spindle translocation is unclear.

There is also evidence that subcortical or cytoplasmic F-actin cables participate in mitotic spindle function in animal cells. This may seem surprising, in that the typical phalloidin-stained mitotic animal cell reveals extensive F-actin localization to the cortex and surface projections such as retraction fibers, but little or no interior actin. However, live imaging of *Xenopus* embryonic epithelia using GFP-Utr-CH showed highly dynamic F-actin cables that formed during mitosis, extended between the spindle poles and cortex, and moved side-to-side in concert with spindle rotations (Woolner et al., 2008; Figure 5). Analysis of F-actin assembly in *Xenopus* egg extracts or intact zebrafish embryos (Field et al., 2011), using either GFP-Utr-CH or Lifeact, revealed that cytoplasmic F-actin cables form in conjunction

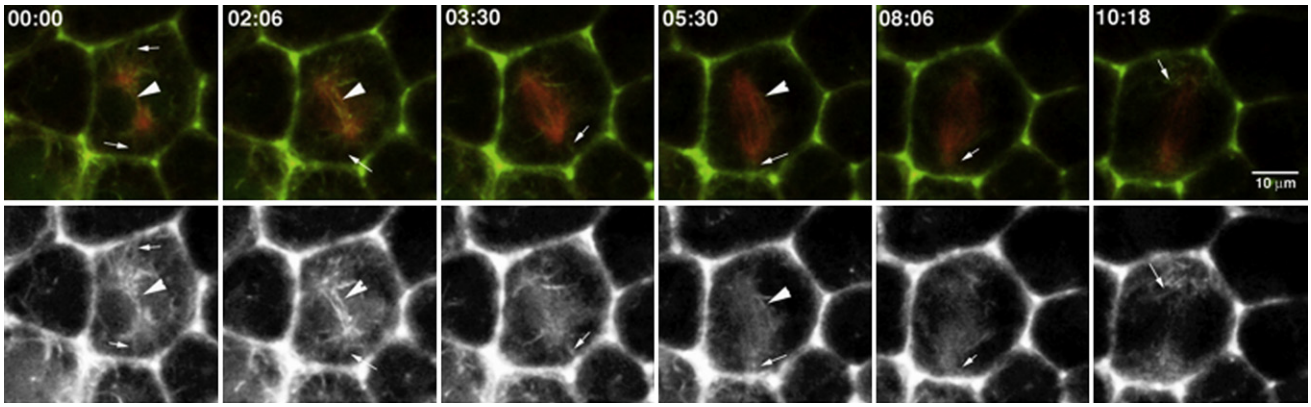

Figure 5. Dynamic F-Actin Associated with Mitotic Spindle in *Xenopus* Embryonic Epithelium
Individual frames taken from time-lapse movie of mitosis in *Xenopus* epithelial cells (reproduced from Woolner et al., 2008). Top frames show double label with microtubules (mCherry-α-tubulin) in red and F-actin (GFP-Utr-CH) in green. Bottom frames show same time points as top frames but with GFP-Utr-CH alone to highlight spindle associated F-actin. Arrows indicate cortical F-actin associated with spindle; arrow heads indicate F-actin within the spindle. Time is min:s.

with entry into mitosis and disappear upon transition into interphase. Further, Mitsushima et al. (2010) found that a bolus of F-actin, dependent on the Arp2/3 complex (which nucleates branched actin networks), forms at the onset of metaphase and circles the subcortex throughout mitosis in cultured mammalian cells. The potential role of this pool of cytoplasmic F-actin in spindle positioning was recently revealed in a fascinating study on the effects of manipulating external forces on spindle orientation in cultured mammalian cells (Fink et al., 2011). Using a combination of micropatterned substrates and microsurgery, the authors demonstrated that modulation of external adhesive forces acting on the cell cortex regulates spindle orientation. Further, they found that the externally applied forces act by modulating the behavior of the oscillating network of cytoplasmic actin described above which dynamically interacts with astral microtubules (Fink et al., 2011). One of the many striking implications of this work is that cortical manipulations influence interior F-actin structures, which, in turn, indicates that previous studies reporting the effects of cortical manipulations should not necessarily be taken to indicate that the effects of these manipulations are limited to the cortex.

Spindle F-Actin and Myosin

While there is a growing, if grudging acceptance that cortical, subcortical, and cytoplasmic F-actin and myosins actively participate in spindle function, potential roles for, and even the existence of, F-actin and myosins within the spindle itself are still viewed with considerable skepticism. In fact, however, there is good evidence that F-actin and myosins are components of spindles in a variety of systems. In plant cells, mitotic spindle F-actin has been convincingly demonstrated using electron microscopy (Forer and Jackson, 1979), phalloidin-staining of fixed samples (Seagull et al., 1987; Traas et al., 1987; Schmit and Lambert, 1987; Yasuda et al., 2005), anti-actin antibodies (Yasuda et al., 2005), and expression of fluorescent actin binding probes (Yu et al., 2006). Collectively these studies show that F-actin is highly dynamic in plants during cell cycle progression (Figure 6). Specifically, the interphase plant cell exhibits cortical F-actin, subcortical F-actin cables, and a network of actin cables around the nucleus, then with the onset of mitosis the subcortical and nuclear cables disassemble and F-actin accumulates at the spindle. By metaphase, F-actin cables extend from the ends of the spindles, where they define a pole-like structure, to the chromosomes. After anaphase onset the phragmoplast, an F-actin-rich network involved in cytokinesis, forms between the separating chromosomes.

Like plant mitotic spindles, phalloidin staining has revealed considerable F-actin in a variety of meiotic spindles including those of maize sporocytes (Staiger and Cande, 1991), insect spermatocytes (Silverman-Gavrila and Forer, 2000), frog oocytes (Weber et al., 2004), and mouse oocytes (Schuh and Ellenberg, 2008). And, like plants, the F-actin is found in the form of cables running from the poles to the spindle midplane. Moreover, these findings have been extended by live imaging in mouse oocytes (Schuh and Ellenberg, 2008; Azoury et al., 2008) where the Utr-CH probe reveals the same distribution of F-actin seen with fixed, phalloidin-stained samples, with F-actin concentrated at the spindle poles and running from the poles to the spindle midplane (Figure 2). In addition to F-actin, myosins have been localized to meiotic spindles, namely, myosin-2 in mouse oocytes (Simerly et al., 1998; Schuh and Ellenberg, 2008), myosin-10 in frog oocytes (Weber et al., 2004), and myosin-5 at the yeast meiotic SPB (Doyle et al., 2009). Moreover, a very recent study has not only shown that a myosin-1 localizes to spindle microtubules in Dictyostelium, this myosin was also found to be essential for spindle stability (Rump et al., 2011).

Nevertheless, for many the key question is whether mitotic spindles in animal cells have spindle F-actin and myosins. This is not merely speciesism, as it can be reasonably argued that in addition to the other obvious differences, plant mitotic spindles and vertebrate oocyte meiotic spindles lack centrosomes and thus may employ distinctly different means of assembly and function than mitotic spindles in animal cells. Surprisingly, while it is currently assumed that animal mitotic spindles do not contain significant amounts of F-actin or myosins, this was not always the case. In the 1970s several groups, using electron microscopy, fluorescent fragments of the skeletal

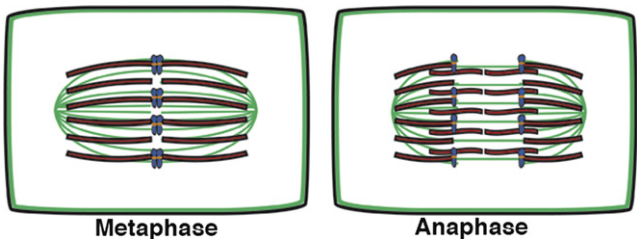

Figure 6. Schematic Diagram Depicting Spindle F-Actin in Mitotic Plant Cells
Cortical F-actin and spindle F-actin are shown in green, microtubules (MTs) in red, the chromosomes in blue, and the kinetochores in orange.

muscle myosin-2 motor domain as a probe for F-actin, fluorescent actin antibodies, or fluorescent myosin-2 antibodies, reported extensive colocalization of F-actin and myosin-2 with the mitotic spindle in several different systems including cultured mammalian cell lines (e.g., Gawadi, 1971; Sanger, 1975; Schloss et al., 1977; Fujiwara and Pollard, 1976; Cande et al., 1977; Herman and Pollard, 1979). Based on such studies, it was suggested that F-actin and myosins might play a variety of roles within the mitotic spindle, including structural support, force production for chromosome movement, a back-up system for chromosome movement, and spindle organization (e.g., Sanger, 1975; Fujiwara and Pollard, 1976; Schloss et al., 1977; Herman and Pollard, 1979). However, the localization of F-actin and myosin-2 in the spindle was subsequently challenged as reflecting an artifact of sample preparation (Aubin et al., 1979), and staining with fluorescent phalloidin failed to yield significant spindle labeling (Barak et al., 1981). Nor was this finding anomalous in that since the report of Barak et al. (1981) phalloidin staining has repeatedly failed to reveal mitotic spindle-associated F-actin in cultured mammalian cells while robustly labeling F-actin in the cell cortex and cell surface projections. Further, live cell studies employing fluorescent F-actin have not revealed spindle-associated F-actin but again clearly reveal cortical F-actin in mitotic animal cells (e.g., Sommi et al., 2011). Moreover, it was demonstrated that suppression of myosin-2 function blocked cytokinesis without preventing spindle assembly and chromosome partitioning (Kiehart et al., 1982). As it became apparent that microtubule-based motors and microtubule turnover itself were strong candidates for basic spindle functions, interest in potential roles for spindle actomyosin was further diminished. Finally, the development of cell-free extract systems showed that F-actin is dispensable for spindle formation in vitro (Lohka and Maller, 1985), in that such extracts are prepared in the presence of cytochalasins, which prevent F-actin assembly.

If the above results, when taken at face value, seem fairly damning to the case for F-actin in animal mitotic spindles, they appear less so when some of the technical challenges of F-actin visualization are considered. Any effort to detect F-actin in fixed samples runs the risk of F-actin loss during fixation and permeabilization, particularly if the pool of F-actin is especially dynamic or labile. This is a particular concern given the reports that detection of spindle F-actin in animal meiotic spindles (Schuh and Ellenberg, 2008) and plant mitotic spindles (Seagull et al., 1987) requires special conditions for preservations. Further, because F-actin is so highly abundant in the cortex, fluorescent F-actin signal from that region may overwhelm any less abundant internal F-actin signal. The same problem confronts visualization of F-actin in living cells, as well as other issues. For example, while the use of fluorescent actin itself (labeled with either a fluorescent protein or a chemical fluorophore) would seem to be a foolproof means for monitoring F-actin in living cells, this approach has its own problems. Fluorescent actin provides less signal-to-noise for detection of F-actin than probes that bind directly to F-actin because at any given moment, the majority of actin in most cells is in the unassembled (G-actin) form. In addition, high levels of fluorescent actin results in cell sickness, although this problem can be overcome simply by titering the amount of expression (e.g., Ballestrem et al., 1998). Indeed, extremely low levels of fluorescent actin expression can be quite useful when exploited using the "speckle" technique, in which protein polymers (F-actin in this case) are detected by visualization of well-separated fluorescent subunits that serve as fiduciary marks (Waterman-Storer et al., 1998).

Somewhat more problematic is the apparent tendency of fluorescent actin to under-report some pools of F-actin. Such underreporting has been directly observed in vitro, with fluorescent actin incorporating poorly into formin-nucleated actin filaments (Kovar et al., 2006). It has also been demonstrated in vivo by the observation that fluorescent (YFP-labeled) actin does not incorporate into the cytokinetic apparatus of fission yeast (which is formed via formin-dependent nucleation) in spite of the fact that this probe incorporates well into cortical actin patches (Wu and Pollard, 2005). That this reflects a physiological deficit in fluorescent actin is indicated by the fact that fluorescent actin cannot substitute for endogenous actin in budding yeast (Wu and Pollard, 2005).

Labeling F-actin in living cells via fluorescent proteins that bind F-actin such as LifeAct (Riedl et al., 2008), Utr-CH (Burkel et al., 2007), or moesin (Edwards et al., 1997) avoids the signal-to-noise problem entailed by use of fluorescent actin itself but has the inevitable problem that high levels of such proteins may stabilize F-actin or compete with binding of endogenous F-actin binding proteins. As with fluorescent actin, titrating expression of fluorescent F-actin binding proteins provides a simple means to overcome these problems. Fluorescent F-actin binding proteins do not apparently underreport formin-templated actin filaments in vivo (for example, see Wu and Pollard, 2005) though they have the potential to underreport any pool of F-actin that assembles and disassembles too quickly to permit probe binding. By combining fluorescent F-actin binding protein with fluorescent actin (Burkel et al., 2007) or fluorescent F-actin binding proteins with apparently different F-actin binding kinetics in the same cell (e.g., LifeAct and Utr-CH; Yoo et al., 2010), it is possible to visualize pools of F-actin that differ in dynamics. In any case, the existence of a given F-actin structure seen in living cells with either fluorescent actin or fluorescent actin binding proteins would ideally be independently confirmed by labeling of fixed samples using fluorescent phalloidin, anti-actin antibodies, or F-actin-specific binding proteins.

In the light of the challenges associated with visualizing F-actin, perhaps the original reports of F-actin-mitotic spindle association in animal cells should be reconsidered. Indeed, in addition to labeling F-actin cables that run between the spindle

poles and cortex (see above), live imaging of mitotic *Xenopus* embryonic epithelial cells using the GFP-Utr-CH probe showed dynamic F-actin cables running from the spindle poles toward the spindle midplane (Woolner et al., 2008; Figure 5). While these findings must be confirmed by analysis of endogenous F-actin in fixed samples, taken with the older studies from cultured cell types, these results argue strongly that more study is warranted.

Likewise, there is good reason to reconsider the potential role of myosins within the spindle. Since the original report of Fujiwara and Pollard (1976), others have also reported myosin localization to mitotic spindles in animal cells. In early *Xenopus* embryos and cultured *Xenopus* epithelial cells, for example, myosin-2 antibodies label mitotic spindles and the label is especially concentrated on the spindle poles (Kelley et al., 1996). This result is not specific to frogs, in that myosin-2A also localizes to mitotic spindles in mouse embryos (Simerly et al., 1998). Further, in cultured mammalian cells, antibodies directed against the phosphorylated (active) form of the myosin-2 regulatory light chain (P-RLC) label the spindle poles (Matsumura et al., 1998). Several different unconventional myosins have also been localized to mitotic spindles, including myosin-5 (Wu et al., 1998) and myosin-10 (Woolner et al., 2008).

Thus, the evidence from different systems demonstrates, to varying degrees, that F-actin and myosins do in fact associate with spindles. However, spindle localization does not prove spindle function, and acquiring such proof is far more difficult than might be imagined for several reasons. First, the available studies have produced widely varying results. With respect to plant mitotic spindles, in some cases, treatment of cells with F-actin-disrupting agents, such as cytochalasins, causes clear spindle phenotypes including disorganization of spindle microtubules and even detachment of chromosomes from the spindle (e.g., Sampson et al., 1996; Sampson and Pickett-Heaps, 2001), while in other cases, similar manipulations have little or no effect on spindle structure or function (e.g., Yasuda et al., 2005). Second, a given pharmacological inhibitor may be less effective in depolymerizing actin in certain situations (e.g., Yasuda et al., 2005) and, even within a species, the same manipulation can produce different effects (for example, see Fabian and Forer, 2007; Xie and Forer, 2008). Third, the interdependency of different M-phase events makes it difficult to determine whether a given phenotype, such as abnormal spindle morphology or chromosome partitioning, results from an early phenotype such as impaired spindle pole separation. Fourth, and perhaps most challengingly, there is no good means to selectively disrupt spindle F-actin or myosins without disrupting other pools of F-actin and myosin. For example, formin ablation results in loss of spindle F-actin within meiotic spindles (e.g., Schuh and Ellenberg, 2008) and prevents spindle translocation to the cortex, but this manipulation also results in loss of cytoplasmic F-actin. Thus, it is presently not possible to strictly test whether both pools of formin-templated F-actin are important, although it seems likely. Likewise, depletion of myosin-10, which localizes to spindles in *Xenopus* embryonic epithelia, results in several distinct spindle phenotypes, including an increase in spindle length (Woolner et al., 2008). Spindle lengthening can be rescued by treatment with latrunculin, which disrupts F-actin, leading to the proposal that myosin-10 within the spindle promotes spindle shortening while F-actin within the spindle or at the cortex promotes spindle lengthening, but because both F-actin and myosin-10 are also localized to the cortex, it is just as likely that all of the phenotypes represent cortical effects (Wühr et al., 2008). Of course, these arguments cut both ways—while a given manipulation surmised to act on spindle F-actin (e.g., Snyder and Cohen, 1995) could be acting on cortical (or cytoplasmic) F-actin, manipulations assumed to be working via perturbation of cortical F-actin could in fact be working via perturbation of spindle (or cytoplasmic) F-actin.

If these considerations make it difficult to directly assign roles to specific pools of F-actin or myosin, there are nevertheless intriguing observations that suggest that not all of the action is at the cortex, subcortex, or cytoplasm. For example, a surprising number of actin regulatory proteins, F-actin binding proteins, and proteins associated with F-actin adhesion, generally concentrated at the cortex in interphase, have been shown to localize to and in some cases regulate mitotic spindle assembly and function in animal cells. Examples include cofilin (Kaji et al., 2008), LIM Kinase (Sumi et al., 2006), cortactin (Wang et al., 2008), zyxin (Hirota et al., 2000), 4.1 (Mattagajasingh et al., 1999), FAK (Park et al., 2009), Rhamm (Maxwell et al., 2003), and integrin kinase (Fielding et al., 2008).

In addition, several groups have reported that myosin-2 inhibition results in spindle phenotypes that are apparently independent of early effects on centrosome separation. For example, Rosenblatt et al. (2004) reported that inhibition of Rho-dependent kinase after formation of normal metaphase spindles resulted in a prolonged mitotic arrest. Similarly, manipulation of myosin light chain kinase (Dulyaninova et al., 2004) or the RLC itself (Komatsu et al., 2000) causes mitotic arrest. Detailed analysis of microtubule and γ-tubulin distribution in these cells revealed apparently normal spindle pole separation but a variety of other defects including, most prominently, abnormally organized spindle microtubules and improper microtubule-kinetochore attachment (Dulyaninova et al., 2004). In addition, it was found that genetic ablation of myosin-2 from mouse cardiocytes results in mitotic failures and spindle abnormalities (Ma et al., 2010). These defects were associated with an increase in acetylated microtubules, an effect not obviously related to, for example, failed spindle pole separation. In some cases, live cell imaging also suggests that disruption of F-actin or myosin function may represent trouble within the spindle itself. For example, depletion of myosin-10 in frog embryonic epithelia results in spindle pole fragmentation (Woolner et al., 2008), a phenotype that resembles failed clustering of supernumary centrosomes in cultured cancer cells subjected to myosin-10 depletion (Kwon et al., 2008). However, time-lapse imaging shows that in the *Xenopus* epithelial cells, the spindle is initially normal when it forms, but subsequently undergoes spindle pole fragmentation, whereas in the cultured cancer cells, the spindles fail to form normal spindles in the first place.

In addition, in fission yeast, actin was recently shown to facilitate SPB and chromosome separation in a process referred to as nuclear fission in a cytokinesis-defective yeast strain that was also treated with a microtubule poison and thus lacked a mitotic spindle (Castagnetti et al., 2010). While it is not clear whether F-actin localizes to the SPBs in this organism, this inference seems reasonable, given the phenotype.

Based on the evidence presented above, we would argue that the case for cortical and cytoplasmic F-actin and myosins in

spindle assembly and function is strong, and the case for spindle F-actin and myosins, though weaker, is nonetheless worth serious consideration. However, it is apparent that clarification of the potential roles of F-actin and myosins in spindle function will ultimately require approaches that have far more power to resolve spatially and temporally the functions of distinct populations of actin and myosin than are typically employed. The necessity for spatial resolution is indicated by the observations described above implicating cortical, subcortical, cytoplasmic, and spindle-associated F-actin and myosins in spindle assembly and function. The necessity for temporal resolution is indicated by the fact that actin and actin-binding proteins are not only now accepted to be components of the nucleus, but also apparently play important roles in a variety of nuclear processes including transcription and chromatin remodeling (Visa and Percipalle, 2010), which could impact mitotic progression either directly or indirectly. Thus, studies in which specific subcellular pools of F-actin or myosins are impaired by precisely timed application of pharmacological reagents, local activation by uncaging, or local inactivation via chromophore-assisted laser inactivation (CALI) or related approaches will prove essential.

The examples of F-actin and myosins contributing to spindle assembly and function prompt many questions, the following of which are particularly intriguing: can specific spindle roles be assigned to specific subcellular pools of F-actin and myosins? What is the cellular and molecular basis of cell-cycle regulation of cytoplasmic F-actin assembly and what, exactly, leads cytoplasmic F-actin to circle the subcortex in an oscillatory fashion? Does this reflect the underlying control of an oscillating kinase system, similar to the Min pathway found in bacteria (Raskin and de Boer, 1999)? Finally, what is the relationship of spindle F-actin and myosins to the so-called spindle matrix, an as yet loosely defined structure thought to surround and support spindles (Johansen et al., 2011)?

ACKNOWLEDGMENTS

We thank our labmates and collaborators, both past and present, and are supported by NIH GM052932 to W.M.B. and by 5F32GM090674 to J.C.S.

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