Introduction

Interactions between microtubules and F-actin are a key feature of many biological processes including cell division, oogenesis, and embryonic morphogenesis (Rodriguez et al., 2003). One of the more fascinating and important examples of such interactions is provided by interaction of mitotic spindles with cortical F-actin. These interactions are required for spindle positioning in yeast (Gundersen and Bretscher, 2003; Gundersen et al., 2004) and are thought to be used for asymmetrical cell division in polarized epithelia (Perez-Moreno et al., 2003) and during orientation of the mitotic spindle in some cultured cell lines (Kaji et al., 2007; Toyoshima and Nishida, 2007). In addition, the F-actin–based motor, myosin-2, has recently been reported to exert force on spindle poles during prophase via cortical flow of anchored microtubules (Rosenblatt et al., 2004).

In the above examples, the key site of actomyosin–microtubule interaction is the cortex, and its major role is thought to be the anchoring or transport of spindle microtubules that extend there. However, whether actomyosin is an important component of the mitotic spindle interior or if actomyosin–microtubule interactions play additional roles in mitotic spindle function and assembly is the subject of an old and intense controversy. A series of fluorescence and electron microscopy studies in the 1970s described the presence of F-actin in the mitotic spindle (Schloss et al., 1977; Forer et al., 1979), and more recent work has found that various actin poisons and myosin inhibitors have varying effects on spindle structure and function (Fabian and Forer, 2007; Forer et al., 2007). However, the studies showing localization were challenged as artifacts of the methods used to prepare the samples (Barak et al., 1981), and inhibition of what was then thought to be the only metazoan actin-based motor, myosin-2, was shown to prevent cytokinesis without having any effect on spindle assembly and function (Kiehart et al., 1982). Finally, and most tellingly, morphologically normal and apparently functional spindles are routinely produced in Xenopus laevis egg extracts under conditions where F-actin assembly is prevented (Mitchison et al., 2005).

In contrast to mitotic spindles, meiotic spindles not only require F-actin for cortical anchoring, but, in many cases, also require F-actin for assembly (Gard et al., 1995; Kim et al., 2000; Sun et al., 2001; Sardet et al., 2002). Consistent with a role for actomyosin in the meiotic spindle, Myo10 (Myo10), an unconventional myosin, was recently shown to localize to meiotic spindles and to be required for their proper assembly (Weber et al., 2004). Myo10 has the unusual property of being able to bind to both F-actin, via a motor domain in its head
cables within the mitotic spindle and show that F-actin and microtubules, is required for proper spindle anchoring, spindle pole integrity, spindle length control, and mitotic progression. Furthermore, we reveal the existence of dynamic actin-myosin interactions in mitotic spindles. We have therefore analyzed the role of both Myo10 and F-actin in mitotic spindle assembly and function in the epithelium of the vertebrate X. laevis. Using a combination of live cell imaging, morpholino (MO)-mediated Myo10 knockdown, and gene replacement, we show that Myo10 localizes to the mitotic spindle and is required for proper spindle anchoring, spindle pole integrity, spindle length control, and mitotic progression. Furthermore, we reveal the existence of dynamic actin cables within the mitotic spindle and show that F-actin and Myo10 play both overlapping and distinct roles during mitosis.

Results

Myo10 localizes to the poles of mitotic spindles in X. laevis embryos

To begin to assess a role for Myo10 in mitosis, we used immunofluorescence in early X. laevis embryos (stages 9–11) using an antibody directed against a small region in the head of Myo10 (Weber et al., 2004). Throughout the cell cycle, Myo10 was found localized at the cell cortex (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200804062/DC1), as expected from previous studies (Berg et al., 2000; Berg and Cheney, 2002). In addition, during interphase, Myo10 localized to the nucleus (Fig. 1 a). However, by metaphase, Myo10 localized to the mitotic spindle, where it was found weakly associated with the entire spindle but concentrated at the spindle poles, and where it remained throughout anaphase and into telophase (Fig. 1 a). Spindle pole localization of Myo10 was also seen in spindles that were assembled in vitro from X. laevis egg extracts (Fig. S1). Double labeling of embryonic spindles for Myo10 and the spindle pole marker γ-tubulin showed that Myo10 localized to a region just beside the γ-tubulin domain (Fig. 1 b). The localization pattern displayed by Myo10 (in the nucleus at interphase and moving to the spindle poles during mitosis) is reminiscent of spindle assembly factors such as TPX2 and NuMA (Meredes et al., 1996; Wittmann et al., 2000). Indeed, triple labeling for α-tubulin, Myo10, and TPX2 demonstrated colocalization of Myo10 and TPX2 in the nucleus and in overlapping domains at spindle poles (Fig. 1 c).

Knockdown of Myo10 causes mitotic spindle defects

The localization pattern of Myo10 suggested that Myo10 might play a role in mitosis. To test this possibility in vivo, we used an MO knockdown approach, which has been widely used as an efficient means of gene suppression in vertebrates (Heasman, 2002). An anti-sense MO oligo was designed to target a 25-nucleotide sequence in the 5′ untranslated region and first 11 coding nucleotides of the X. laevis Myo10 mRNA. The Myo10 MO was microinjected into two cell embryos, and Western blot analysis of lysates made from embryos 24 h after injection showed that Myo10 protein levels were sharply reduced in Myo10 MO-injected embryos (morphants) compared with uninjected controls (Fig. 2 a).

Controls and Myo10 morphants were fixed and stained for α-tubulin at 12, 16, and 24 h after microinjection, and mitotic spindles in the outermost epithelial layer of the embryo were assessed for defects. At 12 h, no discernible mitotic phenotype was seen; however, by 16 h, Myo10 morphants showed a clear multipolar spindle phenotype, which was further enhanced by 24 h (Fig. 2, b and c). At 24 h, an increase in the number of normal bipolar spindles was also seen in the morphants compared with water-injected controls (Fig. 2 c). The increase in bipolar spindles in morphants likely reflects a delay in mitotic progression (see following section). Further analysis of the morphant spindles revealed a third spindle phenotype; Myo10 morphant spindles were significantly longer than controls, both in terms of absolute length (not depicted) and when corrected for cell size (Fig. 2 d). To test if other aspects of spindle organization were affected in morphants, propidium iodide was used to visualize chromosomes. As in controls, most of the chromosomes were localized near to the metaphase plate in morphant metaphase spindles (Fig. 2 e). Moreover, Myo10 morphant spindles in the early stages of the phenotype, exhibiting increased length and one or two extra poles, were observed to have similar chromosomal content to controls (Fig. 2 e), which indicates that these phenotypes were not an indirect result of a previous failed cell division.

To ensure that these phenotypes represent Myo10 depletion, two control experiments were performed: the morphant phenotype was rescued by microinjection of full-length Myo10 (see “The head and tail of Myo10…” and Fig. 5), and embryos were microinjected with a mispair control MO. The control MO differed from the Myo10 MO by just five mispairing nucleotides, and gave a reduced level of Myo10 knockdown (Fig. 2 a) and a correspondingly reduced level of phenotype compared with the morphant (Fig. 2 c), which indicates that Myo10 is the relevant target of the MO. Although these controls demonstrated the specificity of the MO, it was possible that knockdown of Myo10 in the embryo caused spindle defects indirectly by, for example, disrupting nuclear architecture or previous cytokinetic events. To test if the effect on spindle structure was direct, we used the X. laevis egg extract system to assemble spindles in vitro in the absence or presence of a Myo10 antibody. Although control spindles displayed normal bipolar morphology, spindles assembled in the presence of the Myo10 antibody appeared longer and exhibited multipolar phenotypes similar to those in the Myo10 morphants (Figs. 2 f and S2, available at http://www.jcb.org/cgi/content/full/jcb.200804062/DC1).

Myo10 morphant spindles are initially bipolar and then undergo pole fragmentation

The fact that chromosomes localized near the metaphase plate in Myo10 morphant spindles suggested that these spindles initially assembled normally. To explore this possibility, live confocal imaging was used. Using GFP-α-tubulin to visualize
mitotic spindles, we found that it was possible to follow mitosis live in the cells of *X. laevis* embryonic epithelium by time-lapse confocal microscopy (Fig. 3 a and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200804062/DC1). Live imaging revealed four features of the Myo10 morphant phenotype that were not apparent from fixed cell analysis. First, in the initial stages of the morphant phenotype, spindles assembled normally with two poles, although these spindles were longer than controls (Videos 2 and 3). Second, supernumerary poles formed via fragmentation of the original poles, and rather than a wholesale fragmentation, fragmentation was quantal, with only one supernumerary pole forming from each original pole at a time (Fig. 3 b and Videos 2–4). Third, morphant spindles delayed in metaphase for significant periods of time: morphant metaphase took a mean 46.0 ± 8.0 min (n = 5), with 25.4 ± 3.2 min of that time spent as a bipolar spindle, before any pole fragmentation, compared with a metaphase duration of 6.6 ± 0.5 min (n = 4) in controls. The morphant metaphase delay could explain the increase in bipolar spindles seen in the fixed analysis of the morphant (Fig. 2, b and c). Fourth, as the morphant phenotype progressed, cytokinesis failures began to manifest, both in cells where spindles were elongated but remained bipolar (Video 5) and also in those where multipolar spindles, after a long delay, entered...
Figure 2. Knockdown of Myo10 leads to mitotic spindle defects. (a) Western blot showing Myo10 protein levels in uninjected (U), Myo10 MO (MO), and 5-mispair control MO (Ctrl MO) embryos. Lysates were prepared from embryos 24 h after microinjection. (b) Confocal micrographs of α-tubulin staining in embryos microinjected with nuclease-free water or Myo10 MO (MO) fixed at 12, 16, or 24 h after microinjection. (c) Quantification of mitotic spindles in water (H₂O)-, Myo10 MO (MO)-, and 5-mispair control MO (Ctrl MO)-injected embryos 16 and 24 h after microinjection. At 16 and 24 h, Myo10 morphants have significantly more multipolar spindles than the mispair control (red). An increased number of bipolar spindles is seen in the morphant at 24 h, which is suggestive of a delay in mitosis. Error bars represent the standard error of the mean. (d) Box and whisker plots displaying metaphase spindle length in water (n = 92 spindles)- and Myo10 MO (n = 50 spindles)-injected embryos 24 h after injection. Spindle length measurements are shown as a percentage of total cell length to control for differences in cell size. Metaphase spindles in the Myo10 morphant are significantly longer than in water-injected controls. (e) Propidium iodide (red) and α-tubulin (green) staining of control and morphant spindles showing that chromosomes localize to the metaphase plate relatively normally in morphant spindles. (f) Confocal micrographs of α-tubulin–stained spindles assembled in vitro in the presence of either a control antibody or an anti-Myo10 antibody. In control conditions, normal bipolar spindles assemble, whereas the inhibition of Myo10 by antibody addition leads to multipolar spindles. For significance testing, unpaired Student’s t tests were performed: **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
was performed using the MyTH4/FERM domain cassette of Myo10 and full-length TPX2. This assay demonstrated that Myo10 binds directly to TPX2 via its MyTH4/FERM cassette (Fig. 3 e).

Abnormal spindle movement and anchoring in Myo10 morphants

Mitotic spindles in control embryos typically underwent a great deal of abrupt movement in the cell during metaphase, such that they rotated with a jerky motion, dramatically changing their position in very short periods of time (Fig. 4 a and Video 1). In contrast, Myo10 morphant spindles, even before they sprouted supernumerary poles, rotated with a much smoother motion (Fig. 4 b and Videos 2 and 3). Rotation rate measurements showed that control spindles underwent only slight rotation before metaphase, displayed the greatest changes in rotation rate during metaphase, and rotated slower once anaphase began (Fig. 4 c, blue). In comparison, morphant spindles never reached the same extremes in rotation rate but instead gently rotated throughout their extended metaphase; similar movement continued after spindle pole fragmentation and remained unchanged right up to an aberrant anaphase (Fig. 4 c, red).
F-actin and Myo10 function antagonistically to maintain spindle length

The involvement of an actin-based motor in mitosis led us to consider whether actin might be required for mitotic spindle functions. To explore this possibility, morphant and water-injected embryos were treated with low concentrations of latrunculin B (LatB) for 30 min to disrupt F-actin and then immediately fixed and stained for β-tubulin. Treatment of water-injected embryos with LatB caused cells of the epithelium to round up but did not increase the number of bipolar spindles, promote spindle pole fragmentation, or significantly affect mean spindle length (Fig. 6, a, c, and d). However, LatB treatment resulted in impaired anchoring such that spindles oriented perpendicular to the plane of the epithelium, unlike the parallel arrangement in controls (Fig. 6a). Treatment of Myo10 morphants with LatB had no significant effect on the number of bipolar or multipolar spindles (Fig. 6c). However, a startling effect on spindle length was seen when Myo10 morphants were treated with LatB: the longer spindles seen in the morphant were rescued by disrupting F-actin (Fig. 6, b and d), which suggests that Myo10 and F-actin function antagonistically to maintain spindle length.

Live imaging reveals actin cables in and around the mitotic spindle

The role and distribution of F-actin in the mitotic spindle has long been controversial (see Introduction), but the finding that F-actin and Myo10 together played a role in controlling spindle length prompted us to reinvestigate F-actin distribution in mitotic cells. To visualize F-actin in living embryos, we used a recently

The head and tail of Myo10 have distinct mitotic roles

The N-terminal head domain of Myo10 binds to F-actin (Homma et al., 2001), whereas the C-terminal tail contains a MyTH4/FERM domain cassette that binds microtubules (Weber et al., 2004). To determine if the different mitotic functions of Myo10 were mediated through different regions of the myosin, rescue experiments were performed (see Fig. 5 a for schematics of rescue constructs). Microinjection of full-length Myo10 (GFP-Myo10) into morphants gave a strong rescue of the key mitotic phenotypes: GFP-Myo10 reduced the increased number of bipolar spindles seen in the morphant (Fig. 5, b and c), reduced spindle pole fragmentation (Fig. 5, b and c), and reduced spindle length (Fig. 5 d). In contrast the GFP–heavy meromyosin (HMM) construct, an HMM-like fragment that contains the myosin motor, regulatory IQ domains, and coiled coil domains of Myo10, reduced the number of bipolar spindles (Fig. 5 c) and shortened spindle length (Fig. 5 d) but did not rescue the multipolar phenotype (Fig. 5 c). Remarkably, the GFP-Myo10-IQT construct, which lacks the motor domain but contains the tail, IQ, and coiled coil domains, gave the opposite result: the multipolar phenotype was significantly rescued but bipolar spindle numbers were not reduced (Fig. 5 c) and the spindle length phenotype was only partially rescued (Fig. 5 d). These results indicated that the actin-binding head was required for proper progression through mitosis and for maintaining spindle length, two functions that may be linked, whereas the microtubule-binding tail was required for spindle pole integrity.
A previous study indicated that Myo10 has the ability to organize F-actin (Tokuo et al., 2007), and to explore this possibility, we imaged F-actin live in Myo10 morphants. However, we saw no obvious change in cortical or spindle-associated F-actin in the Myo10 morphants, although it is possible that there are subtle changes to F-actin organization that are beyond the sensitivity of our current imaging methods. In particular, F-actin cables could still be seen surrounding the morphant spindle as it assembled, just as was observed in controls (Fig. 7 d and Video 9, available at http://www.jcb.org/cgi/content/full/jcb.200804062/DC1).

Discussion

In this study, we show that Myo10 localizes to the mitotic spindle pole and plays several important roles in mitosis: spindle anchoring, maintenance of spindle pole integrity, spindle length control, and mitotic progression. Surprisingly, the relationship between Myo10 and F-actin varies depending on the role in question, with Myo10 functioning independently of F-actin to maintain spindle pole integrity, and Myo10 and F-actin working antagonistically to control spindle length.

Figure 5. Rescue experiments reveal that the head and tail of Myo10 mediate different aspects of Myo10 function in mitosis. (a) Schematic diagram of the constructs used in Myo10 morphant rescue experiments. (b) Low-magnification images of α-tubulin staining in rescue experiment embryos. For these experiments, embryos were microinjected with water or Myo10 MO along with RNA encoding each of the Myo10 constructs shown in panel a. (c) Quantification of bipolar and multipolar spindles in rescue experiment embryos. n = 18, 47, 22, 16, and 12 embryos for water, MO, MO + GFP-Myo10, MO + GFP-Myo10-HMM, and MO + GFP-Myo10-IQT, respectively. Error bars represent the standard error of the mean. (d) Box and whisker plots of spindle length measurements in rescue experiment samples. Spindle length was calculated as a percentage of total cell length to allow for variation in cell size. n = 109, 141, 136, 115, and 87 spindles for water, MO, MO + GFP-Myo10, MO + GFP-Myo10-HMM, and MO + GFP-Myo10-IQT, respectively. For significance testing, unpaired Student’s t-tests were performed: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
the spindle through interactions with F-actin fits well with the fact that actin is known to play a vital role in positioning the spindle during oriented cell division (Gundersen and Bretscher, 2003; Rodriguez et al., 2003; Gundersen et al., 2004) and the recent finding that Myo10 is required for spindle orientation in cultured mammalian cells (Toyoshima and Nishida, 2007).

A second function of Myo10 during mitosis is to maintain spindle pole integrity, as disrupting Myo10 either in vitro or in vivo leads to the formation of multipolar spindles. An unusual aspect of this phenotype is that spindle poles first appear to assemble normally and only fragment subsequently. This suggests that Myo10 is not required to establish bipolarity during spindle assembly but instead acts to stabilize spindle poles as mitosis progresses, although it is also possible that the late onset of the multipolar phenotype simply reflects a slow loss of Myo10 protein in morphant cells. It is interesting to note that a similar late-onset pole fragmentation phenotype is seen when Borealin, a member of the chromosomal passenger complex, is depleted in mammalian cells (Gassmann et al., 2004). Exploring a link between Myo10 and this complex could be a fruitful area for further study. Strikingly, the function of Myo10 in the spindle pole is independent of F-actin, as LatB treatment has no effect on spindle pole integrity either in control or morphant embryos. Furthermore, injection of LatB into mitotic embryos does not affect the number of bipolar or multipolar spindles. LatB treatment does significantly rescue the increased spindle length seen in Myo10 morphants (n = 82, 104, 86, and 59 spindles for control, LatB, MO, and MO + LatB, respectively). For significance testing, unpaired Student's t tests were performed: ****, P < 0.0001.

Our findings indicate that Myo10 and F-actin both help to anchor the spindle during mitosis. First, spindles in Myo10 morphants undergo strikingly different movements to controls, with morphant spindles moving smoothly compared with the rapid changes in rotation rate displayed by control metaphase spindles. Second, disrupting F-actin in embryos leads to spindle positioning defects, with spindles orienting perpendicular, rather than parallel, to the cortex. The fact that knocking down Myo10 eliminates jerky spindle movement suggests that Myo10 functions in spindle anchoring by providing transient links between the spindle and the cortex through brief, high-affinity interactions with F-actin, generating a stop-start movement. It may seem counterintuitive that disrupting anchoring would lead to a reduction in rotation rate, but this phenomenon has also been seen in the Caenorhabditis elegans embryo, where knockdown of dynein, a microtubule motor that is located at the cell cortex and pulls on spindle microtubules, leads to an attenuation of spindle oscillation (Pecreaux et al., 2006). It is unclear whether Myo10’s spindle anchoring capacity is mediated through cortically localized Myo10, possibly functioning in concert with cortical dynein, or through Myo10 associated with the spindle. However, it is interesting to note that because we see actin cables extending between the cortex and the spindle pole, the latter possibility is highly plausible. The notion that Myo10 acts to anchor the spindle through interactions with F-actin fits well with the fact that actin is known to play a vital role in positioning the spindle during oriented cell division (Gundersen and Bretscher, 2003; Rodriguez et al., 2003; Gundersen et al., 2004) and the recent finding that Myo10 is required for spindle orientation in cultured mammalian cells (Toyoshima and Nishida, 2007).

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of GFP-Myo10-IQT, a Myo10 tail construct that lacks the actin-binding myosin head, specifically rescues the pole fragmentation phenotype in Myo10 morphants. A possible explanation for Myo10 tail function in spindle pole integrity is that domains in the tail act to recruit and/or retain pole proteins such as TPX2, NuMA, or γ-tubulin at the pole. Consistent with this idea, disruption of TPX2 has been previously shown to generate multipolar spindles (Garrett et al., 2002), and we find that TPX2 fails to localize properly to the spindle pole in Myo10 morphants. In addition, Myo10 can interact directly with TPX2 via its MyTH4/FERM domain cassette. However, it remains unclear how TPX2 functions in spindle pole focusing, and our data do not rule out the possibility that TPX2 is instead required to recruit Myo10 to the pole to ensure pole integrity; resolving this issue will be an important line of further investigation. The upstream control of TPX2 is much better understood, as, like other spindle assembly factors, TPX2 is held at the nucleus during interphase through an interaction with the nuclear importins and is released from this by Ran GTPase as mitosis begins (Gruss and Vernos, 2004). It is interesting to note the similarities between the localization patterns of Myo10 and TPX2 and consider if Myo10 localization may also be regulated by the Ran GTPase pathway. Previous work has shown that the tail of Myo15, a MyTH4/FERM myosin, binds to the nuclear importin, importin-α (Liu et al., 2008), and, intriguingly, we also find by yeast two-hybrid assay that the MyTH4/FERM domain cassette of Myo10 interacts with importin-α (unpublished data), which suggests that the localization of Myo10 may be controlled in a similar manner to spindle assembly factors.

Figure 7. Live imaging in X. laevis embryos reveals actin cables around the mitotic spindle, which are unaffected in Myo10 morphants. (a) Images taken from a video showing F-actin organization in a control mitotic spindle (see Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200804062/DC1) using mCherry-γ-tubulin (red) to visualize the spindle and the GFP-Utr-CH probe (green) to visualize F-actin. Highly dynamic F-actin cables surround the spindle as it assembles (t = 0:00–2:00, arrows) and are concentrated around the poles later in mitosis, especially during anaphase (t = 11:00 and 13:00, arrows). (b) An enlarged view of the uppermost spindle pole from panel a showing that F-actin (GFP-Utr-CH, green) is concentrated around the pole, with some F-actin cables appearing to emanate from the pole (t = 0:00 and 0:06, arrows) and others from the cortex (t = 0:12, arrows). (c) Stills taken from a video of a second control spindle (see Video 8) demonstrating that the assembly of F-actin cables between the spindle and the cell cortex coincide with spindle movement (t = 0:00–0:18, arrows) and concentrate as a pole is drawn toward the cortex (t = 0:24 and 0:30, arrows). (d) Images taken from a video showing F-actin (GFP-Utr-CH, green) organization during Myo10 morphant spindle assembly (see Video 9). F-actin cables associate with the morphant spindle as it assembles (arrow), just as occurs in controls. (e) Stills of a multipolar spindle in a Myo10 morphant (see Video 9) demonstrating that F-actin associates with each of the poles and follows the motion of the spindle (arrows). In each panel, time stamps indicate time in minutes and seconds.
A third role for Myo10 in mitosis is regulation of spindle length. Specifically, Myo10 depletion results in spindles that are significantly longer than controls, both in absolute terms and as a function of cell size. Remarkably, this increased spindle length is rescued by disruption of F-actin with LatB, indicating that Myo10 and F-actin somehow play opposing roles in mitotic spindle length control. How can these findings be reconciled? The simplest explanation is that actin filaments participate in mutually opposing mechanisms that control spindle length: one mechanism that lengthens spindles and is independent of Myo10 and one that shortens spindles and is dependent on Myo10 (Fig. 8). When Myo10 is depleted, the former predominates, causing spindle lengthening; when F-actin is disrupted by latrunculin treatment either with or without Myo10 depletion, both mechanisms are neutralized, so spindle lengths are similar to controls. This model is attractive not only because it explains the results of the current study, but also because it might account for previous findings in X. laevis egg extracts showing that F-actin disruption has no obvious effect on spindle length (Mitchison et al., 2005). Curiously, in the current study, addition of Myo10 antibodies to extracts resulted in spindles that appeared longer than controls, in spite of the fact that cytochalasin D was present. Although this might simply reflect the apparent fragmentation of the poles, there is another potential explanation. That is, previously, we have shown that microtubules in extracts interact with dynein immobilized on the glass substrate (Waterman-Storer et al., 2000), and it may be that this interaction produces an outward pulling force in the extract spindles, which is exaggerated when Myo10 is depleted.

Assuming one or more aspects of the above model are correct, it will be essential in future studies to determine how exactly the F-actin–dependent lengthening and shortening mechanisms work. Two general, nonexclusive possibilities should be considered. First, it is possible that the mechanisms work in a relatively direct manner, with the lengthening mechanism being underpinned by pulling forces on the poles via F-actin and dynein (Sharp et al., 2000) and/or myosin-2 (Rosenblatt et al., 2004), whereas the poles are pulled together by Myo10 acting on the spindle-associated F-actin described here. Alternatively, as considerable evidence links spindle length control to tubulin turnover (Goshima et al., 2005; Mitchison et al., 2005), it is also possible that the F-actin–dependent spindle length control mechanisms function more indirectly, by modulating microtubule assembly and disassembly within the spindle. The F-actin cables that we see associated with the mitotic spindle could contribute to either of these mechanisms, and this will be an important area for further investigation.

The extent to which the various Myo10 roles described above are interrelated must be considered. The differential dependence on F-actin indicates that at least some aspects of the phenotypes can be considered separately. However, it is also likely that some of them are coupled. For example, it seems plausible that the metaphase delay is related to the spindle length defect, in that it is not hard to imagine that improper length control, and any subsequent loss of tension, would elicit the spindle assembly checkpoint (Nicklas et al., 1995; Zhou et al., 2002). Further, it is also probable that at least some of the deficits that result from Myo10 depletion feed back on each other. That is, in videos where the phenotype is just starting to become manifest, excessive spindle length and metaphase delay are apparent before excessive spindle pole fragmentation (Fig. 2). However, at least some of the cells with elongate spindles fail to complete cytokinesis without having obviously fragmented their poles (e.g., Video 5). Such cells would thus start the subsequent mitosis with additional poles. This condition would, presumably, further delay metaphase, provide additional material for pole fragmentation, and increase the probability of another failed cytokinesis. Consequently, in just one or two cell cycles, cells could go from an
initially modest phenotype, wherein spindles have just two extra poles, to the severe phenotype in which upwards of 15 extra poles are apparent (e.g., Fig. 2, b and c).  

Materials and methods

Egg procurement and in vitro fertilization

Adult X. laevis females were first primed for ovulation by injection with 50 units of human chorionic gonadotrophin (HCG; MP Biomedicals) into the dorsal lymph sac 4–7 d before use. Eggs were then injected with 1x MMR (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM Hepes, pH 7.4) and fertilized by adding macerated testes. 30 min after fertilization, embryos were dejellied in 2% cysteine (1x MMR, pH 7.8) and rinsed five times with 1x MMR and five times with 0.1x MMR. Embryos were cultured in 0.1x MMR at 17°C.

Embryo microinjection

RNA for microinjection was made as described previously using pCS2-EGFP, pCS2-mCherry, or pStain vectors (Sokac et al., 2003). Microinjections were delivered using a PLI-picojector (Medical Systems), with embryos submerged in 0.1x MMR + 5% Ficoll. Myo10 MO (MO sequence: 5’TATTCCTCAGTTCCTGTCCTGTC-3’; Gene Tools, LLC) or 5-mispair control MO (5’TATTCGTTATTCCTGCTC-3’) was used to monitor Myo10 localization. All other mRNA constructs were synthesized from plasmid templates by in vitro transcription and purified on a Sepharose CL-6B column. Embryos were microinjected at the four-cell stage with 2.5 nl of Myo10 MO (0.5 mM) or nuclease-free water. For morphant rescue experiments, both cells of two-cell embryos were microinjected with 5 nl of RNA for microinjection into two- or four-cell embryos at a middle concentration, 1 mM) into both cells at the two-cell stage and incubated 12–24 h in 0.1x MMR before processing for live imaging, fixation, or Western blot analysis.

In vitro spindle assembly

Cytostatic factor (CSF)-resistant X. laevis egg extracts were prepared as described previously (Murray, 1991). X. laevis sperm chromatin was added and the extracts were subsequently cycled into interphase by the addition of 400 μM Ca²⁺. After 60 min, an equal volume of mitotic extract was added and the extracts were subsequently microinjected at the four-cell stage in all cells with 2.5 ml of Myo10 MO (0.5 mM) or nuclease-free water. For morphant rescue experiments, both cells of two-cell embryos were microinjected with 5 nl of RNA for the GFP-tagged Myo10 constit. (0.5 mg/ml) and then subsequently microinjected at the four-cell stage with 2.5 nl of Myo10 MO (0.5 mM) or nuclease-free water. After microinjection, embryos were incubated at 17°C for 12–24 h in 0.1x MMR before processing for live imaging, fixation, or Western blot analysis.

Immunofluorescence

For immunofluorescent analysis of microtubules, only embryos were processed as described previously (Danichik et al., 1998). For all other staining, the methanol postfix step was omitted from this protocol. Antibodies used were: anti-α-tubulin (DM1A; 1:200 dilution; Sigma-Aldrich), 5 μg/ml anti-Myo10 (Weber et al., 2004), anti-TX2 (1:800; O’Brien and Wiese, 2000), mouse anti-α-tubulin (GTUBB; 1:200; Sigma-Aldrich), and 10 μg/ml rabbit anti-γ-tubulin (Kaoing and Borisy, 2000). To visualize chromatin, embryos were fixed and processed as above, omitting the methanol postfixation, and then permeabilized for 5 min at room temperature in PBS + 0.5% Triton X-100, equilibrated in 2x SSC (0.3 M NaCl) and 0.03 M sodium citrate, pH 7.7, treated with 100 μg/ml DNase-free RNase in 2x SSC for 20 min at 37°C, and then incubated with 5 μM propidium iodide (Invitrogen) in 2x SSC for 5 min at room temperature.

LatB treatment of embryos

Embryos were microinjected with nuclease-free water or 100 μM LatB (needle concentration, 1 mM) into both cells at the two-cell stage and incubated at 17°C for 20 h. Embryos were then incubated for 30 min at room temperature in 0.1% DMSO or 2.5 μM LatB in 1x MMR. All embryos were fixed immediately after treatment and stained for α-tubulin as described in the previous paragraph.
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