

Myosin and the PAR proteins polarize microfilament-dependent forces that shape and position mitotic spindles in *Caenorhabditis elegans*

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In *Caenorhabditis elegans*, the partitioning proteins (PARs), microfilaments (MFs), dynein, dynactin, and a nonmuscle myosin II all localize to the cortex of early embryonic cells. Both the PARs and the actomyosin cytoskeleton are required to polarize the anterior-posterior (a-p) body axis in one-cell zygotes, but it remains unknown how MFs influence embryonic polarity. Here we show that MFs are required for the cortical localization of PAR-2 and PAR-3. Furthermore, we show that PAR polarity regulates MF-dependent cortical forces applied to astral microtubules

(MTs). These forces, which appear to be mediated by dynein and dynactin, produce changes in the shape and orientation of mitotic spindles. Unlike MFs, dynein, and dynactin, myosin II is not required for the production of these forces. Instead, myosin influences embryonic polarity by limiting PAR-3 to the anterior cortex. This in turn produces asymmetry in the forces applied to MTs at each pole and allows PAR-2 to accumulate in the posterior cortex of a one-cell zygote and maintain asymmetry.

Introduction

First identified in the nematode *Caenorhabditis elegans* (Kemphues et al., 1988), the conserved partitioning proteins (PARs)* are required for cell polarity in many animal cell types (for review see Doe and Bowerman, 2001; Wodarz, 2002). In the one-cell stage *C. elegans* embryo, the PDZ domain protein PAR-3 and the Ring finger protein PAR-2 concentrate in complementary anterior and posterior cortical domains, respectively. Both are required to specify the anterior-posterior (a-p) body axis and to orient and position mitotic spindles relative to the a-p axis (Kemphues et al., 1988; Cheng et al., 1995; Etemad-Moghadam et al., 1995; Boyd et al., 1996) (Fig. 1, a and b).

One a-p asymmetry regulated by PAR-2 and PAR-3 appears during telophase of the first mitosis when the initially spherical posterior centrosome changes shape to form a disc, whereas

the anterior centrosome remains spherical (Hill and Strome, 1988; Cheng et al., 1995; Keating and White, 1998). Previous observations of early embryos made using Nomarski DIC microscopy have suggested that PAR-3 inhibits flattening of the anterior spindle pole, whereas PAR-2 prevents this inhibition from occurring at the posterior pole (Cheng et al., 1995). Centrosome flattening may reflect an asymmetry in forces applied to the centrosomes through astral microtubules (MTs) that contact the cell cortex during mitosis. This asymmetry in force displaces the first mitotic spindle toward the posterior pole and generates lateral rocking motions of the posterior spindle pole during the asymmetric first division of a one-cell zygote (Cheng et al., 1995; Grill et al., 2001; Tsou et al., 2002).

Results and discussion

SPD-5 and centrosome shape

To examine centrosome shape, we stained fixed wild-type and *par* mutant embryos with antibodies that recognize a centrosomal protein called SPD-5 (Hamill et al., 2002) (Fig. 1, c, f, and i). In wild-type, the posterior centrosome flattened late in mitosis, whereas the anterior centrosome remained spherical (Fig. 1 c). In *par-2(lw32)* mutant embryos, PAR-3 spread around the posterior cortex, and both centrosomes remained spherical, like the anterior centrosome in a wild-type zygote (Fig. 1, d–f) (Cheng et al., 1995; Etemad-Moghadam et al.,

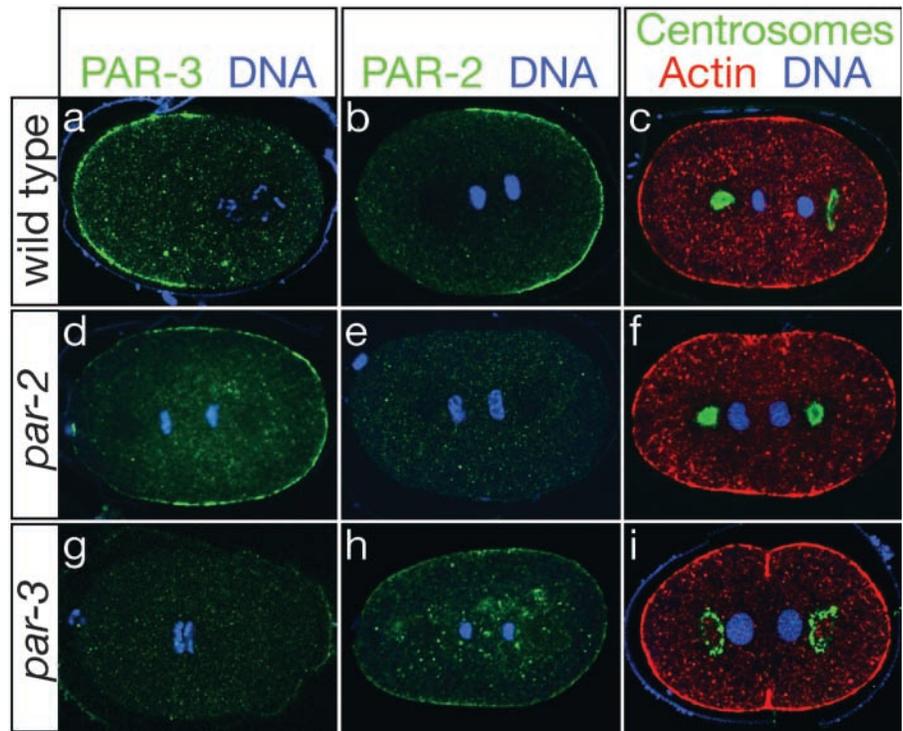
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*Abbreviations used in this paper: a-p, anterior-posterior; DHC, dynein heavy chain; DNC, dynactin component p150glued; LatA: latrunculin A; MF, microfilament; MLC, myosin II regulatory light chain; MT, microtubule; NCC, nucleocentrosomal complex; NMY, nonmuscle myosin II heavy chain; PAR, partitioning protein; RNAi, double stranded RNA-mediated interference.

Key words: dynein ATPase; microfilaments; myosin type II; mitotic spindle apparatus; cell polarity

Figure 1. PAR proteins and mitotic spindle polarity in the *C. elegans* zygote.

(a and b) In wild-type embryos, PAR-3 accumulates at the anterior cortex, whereas PAR-2 is localized to the posterior. (c) The posterior centrosome flattens in telophase. (d–f) In *par-2(lw32)* mutant embryos, PAR-3 spreads around much of the posterior cortex, PAR-2 is undetectable at the cortex, and both mitotic spindle poles remain symmetrical and rounded. (g–i) In *par-3(it71)* mutant embryos, PAR-2 encircles the embryo and both centrosomes flatten. In this and all subsequent figures, embryos are oriented with their anterior pole to the left.



1995). Conversely, PAR-2 accumulated throughout the cortex in *par-3(it71)* mutants, and both centrosomes flattened to resemble a wild-type posterior centrosome (Fig. 1, g–i) (Cheng et al., 1995; Boyd et al., 1996).

Centrosome flattening requires microfilaments

Disruption of MF assembly results in a-p polarity defects similar to those caused by mutations in *par-2*. In wild-type embryos treated with cytochalasin D (Hill and Strome, 1988) or latrunculin A (LatA; Fig. 2 c; eight out of nine embryos), neither centrosome flattened. The failure of either pole to flatten could result from mislocalized PAR-3 inhibiting flattening at both poles as in *par-2* mutants (Cheng et al., 1995). Moreover, MFs might be required for cortical localization of the PAR proteins, with such localization being important for their function. Therefore, we examined the localization of PAR-2 and PAR-3 in embryos exposed to LatA. We found that PAR-2 and PAR-3 both require intact MFs to localize to the cortex. Both were undetectable at the cortex, or present at severely reduced levels, in the presence of LatA (Fig. 2, a and b; $n \geq 5$ for each; see Materials and methods). PAR-2 accumulated around the centrosomes of LatA-treated embryos as was observed recently in *pod* mutants with defects in a-p polarity (Rappleye et al., 2002). We also examined centrosome flattening and PAR localization in embryos with reduced levels of the profilin PFN-1, which we have recently shown is required for the assembly of cortical MFs (Severson et al., 2002) (Fig. 2 g). Consistent with our findings in LatA-treated embryos, the posterior centrosome failed to flatten in embryos depleted of PFN-1 using dsRNA-mediated gene silencing, or RNAi (Fig. 2 g), and PAR-2 was undetectable at the cortex but instead localized around centrosomes (Fig. 2 f; 10 out of 12 embryos). Although PAR-3 was always detected at the cortex in PFN-

1-depleted embryos, it was present at much reduced levels compared with wild-type embryos fixed on the same slides (Fig. 2 e; six out of six embryos). The remaining cortical PAR-3 may simply reflect residual MF assembly because low levels of cortical F-actin still assemble in embryos with reduced levels of profilin (Severson et al., 2002). We conclude that centrosome flattening and the cortical localization of PAR-2 and PAR-3 all require an intact MF cytoskeleton.

PAR-3 prevents flattening of the anterior centrosome in wild-type embryos and of both the anterior and posterior centrosomes in *par-2* mutants. In LatA-treated embryos, PAR-3 is not present at the cortex, but cytoplasmic PAR-3 could still function to prevent centrosome flattening. Therefore, we examined centrosome shapes in *par-3* mutants exposed to LatA. We found that both centrosomes, which are flattened in *par-3* single mutants and in *par-2 par-3* double mutants, were spherical as in LatA-treated wild-type embryos (Fig. 2 d; $n = 6$). Both centrosomes were also spherical in *pfn-1; par-3* double mutant embryos (Fig. 2 h; $n = 10$). We conclude that in addition to being required for the cortical localization of PAR-2 and PAR-3, MFs are required for the process of centrosome flattening itself. PAR-2 and PAR-3 together restrict flattening to the posterior pole but are not required for production of the forces that underlie this process.

We also tested whether MFs are required for spindle orientation in two-cell stage embryos. In wild-type, the centrosomes of both two-cell stage blastomeres initially are aligned orthogonal to the a-p axis. During mitotic prophase, the nucleus and its associated centrosomes (nucleo-centrosomal complex [NCC]) in the posterior blastomere rotate 90°. A mitotic spindle subsequently assembles parallel to the a-p axis in this cell, whereas the spindle in the anterior cell remains transverse (Hyman and White, 1987)

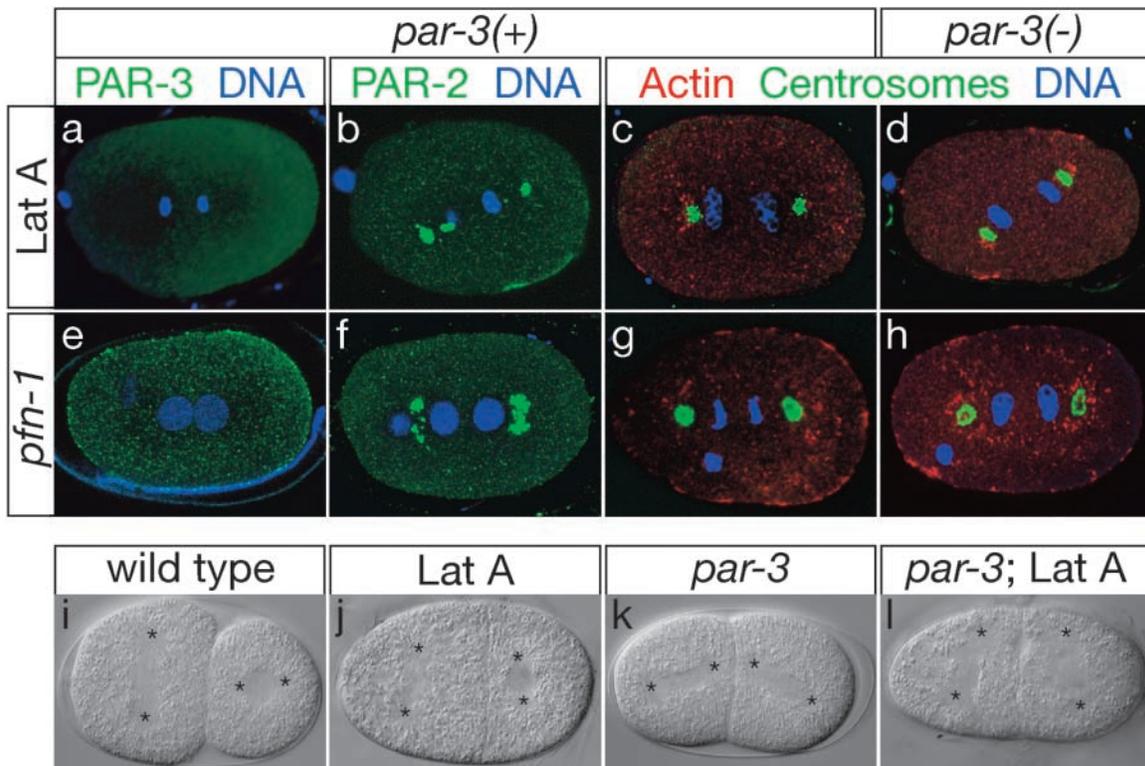


Figure 2. Intact MFs are required for cortical PAR localization, centrosome flattening, and mitotic spindle orientation. (a and b) Disrupting MF assembly with LatA results in a loss of cortical PAR-3 and PAR-2. (e and f) Cortical PAR protein levels also are reduced in *pfn-1(RNAi)* embryos. (c and g) Cortical MF assembly is disrupted in LatA-treated and *pfn-1* mutant embryos (red). Both the anterior and posterior centrosomes remain rounded (green), and the first mitotic spindle does not become posteriorly displaced. (d and h) Centrosomes remain rounded in *pfn-1(RNAi)*; *par-3(it71)* double mutants and in *par-3(it71)* mutants treated with LatA, suggesting that PAR-3 does not inhibit centrosome flattening in embryos with disrupted F-actin. Instead, we suggest that intact MFs are required for centrosome flattening itself. (i–k) Mitotic spindle orientation at the two-cell stage. Asterisks indicate the position of spindle poles. (i) In wild-type embryos, the posterior spindle lies along the a-p axis, perpendicular to the anterior spindle. (j) Both spindles are transverse in LatA-treated embryos. (k) Both spindles are parallel to the a-p axis in *par-3(it71)* mutant embryos. (l) Both spindles are transverse in *par-3(it71)* embryos treated with LatA.

(Fig. 2 i). In *par-3* mutants, both NCCs rotate (Fig. 2 k; eight out of ten embryos), whereas both remain transverse in *par-2* mutants (Kemphues et al., 1988). However, both rotate in *par-2 par-3* double mutant embryos, indicating that neither PAR protein is required for NCC rotation (Cheng et al., 1995). As shown previously in experiments using cytochalasin D (Hyman and White, 1987), we observed that the posterior NCC failed to rotate in wild-type two-cell stage embryos treated with LatA (Fig. 2 j; $n = 6$; see Materials and methods). Similarly, both NCCs failed to rotate in two-cell stage *par-3* mutant embryos exposed to LatA (Fig. 2 l, $n = 6$). Thus, MFs mediate changes both in spindle pole shape at the one-cell stage and in spindle orientation at the two-cell stage, with PAR-2 and PAR-3 regulating where these changes occur.

Myosin II is not required for centrosome flattening

We next examined how myosin II influences the MF-dependent forces that flatten spindle poles. Depletion of the non-muscle myosin II heavy chain (NMY)-2 or of the myosin II regulatory light chain (MLC)-4 (Guo and Kemphues, 1996; Shelton et al., 1999) results in embryonic polarity defects similar to those in LatA-treated embryos: the first mitotic spindle remains centrally positioned and both spindle poles remain spherical (Fig. 3, b and f). However, one difference is

that PAR-3 accumulates around both the anterior and posterior cortex in embryos depleted of either myosin II subunit (Guo and Kemphues, 1996; Shelton et al., 1999), whereas PAR-3 is not present at the cortex in LatA-treated embryos (see above). In contrast to PAR-3, PAR-2 was usually present in a reduced cortical patch in mutant embryos depleted of NMY-2 or MLC-4, (Fig. 3, a and e; five out of eight *nmy-2* and six out of eight *mlc-4* embryos) or was undetectable at the cortex (three out of eight *nmy-2* and two out of eight *mlc-4* mutants) (Shelton et al., 1999). Thus, unlike MFs, neither NMY-2 nor MLC-4 are required for PAR-3 to associate with the cortex, but they are required for the polarized distribution of cortical PAR-3 and for the posterior cortical localization of PAR-2.

We next asked whether the PAR-3 present throughout the cortex in myosin-depleted embryos inhibits flattening at both spindle poles. We found that both centrosomes flattened in *nmy-2(RNAi)*; *par-3(it71)* ($n = 24$) and *mlc-4(RNAi)*; *par-3(it71)* double mutant embryos (Fig. 3, d and h; $n = 7$). Thus, NMY-2 and MLC-4 are dispensable for the MF-dependent forces that underlie centrosome flattening. Instead, myosin II appears to facilitate the establishment of normal, complementary domains of PAR-2 and PAR-3, which in turn regulate the distribution of the forces that influence spindle shape and position.

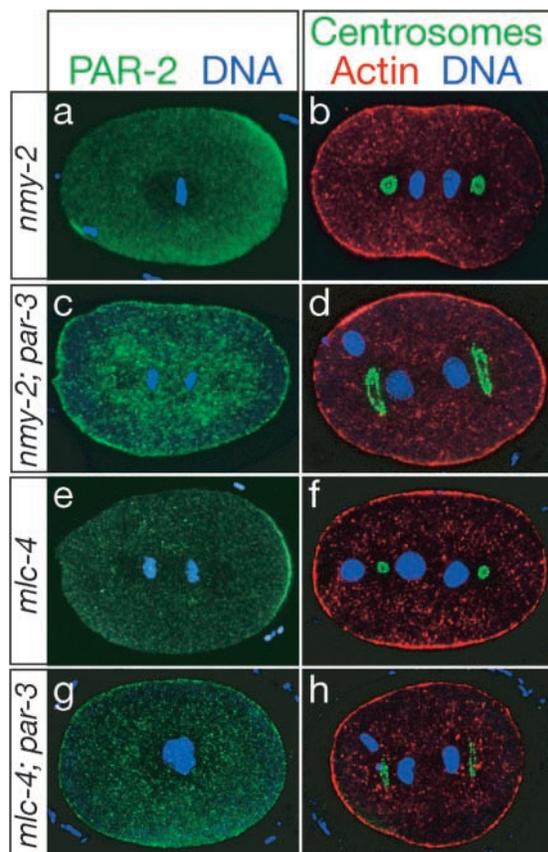


Figure 3. Nonmuscle Myosin II is required to establish a normal PAR boundary, but is not required for centrosome flattening. (a and e) PAR-2 usually accumulates in a small posterior patch in *nmy-2(RNAi)* and *mlc-4(RNAi)* mutant embryos. (b and f) Both centrosomes are round in *nmy-2(RNAi)* and *mlc-4(RNAi)* embryos. (d and h) Both centrosomes flatten in *nmy-2(RNAi); par-3(RNAi)* and *mlc-4(RNAi); par-3(RNAi)* embryos. (c and g) PAR-2 extends around the anterior of *nmy-2(RNAi); par-3(RNAi)* and *mlc-4(RNAi); par-3(RNAi)* embryos.

Myosin II restricts PAR-3 to the anterior cortex

As described above, PAR-3 accumulates around the cortex of myosin-depleted embryos, whereas PAR-2 and PAR-3 localize in mutually exclusive cortical domains in wild-type zygotes. Myosin II could influence the localization of PAR-2 and PAR-3 by facilitating expansion of the PAR-2 domain, thereby restricting PAR-3 to the anterior cortex. Alternatively, myosin might limit PAR-3 localization to the anterior hemisphere, thus permitting expansion of the PAR-2 domain. To distinguish between these two models, we examined the localization of PAR-2 in NMY-2-depleted and in MLC-4-depleted *par-3* mutant embryos. In both cases, we found that PAR-2 was present throughout the cortex, suggesting that neither myosin II subunit is required for cortical localization or expansion of PAR-2 (Fig. 3, c and g; $n \geq 5$ for each double mutant). Instead, myosin appears to restrict PAR-3 to the anterior, with ectopic PAR-3 preventing PAR-2 accumulation at the cortex in myosin II-depleted embryos.

Centrosome flattening requires dynein and dynactin

The results described above suggest that MFs either recruit or activate a cortical motor protein that pulls on astral MTs

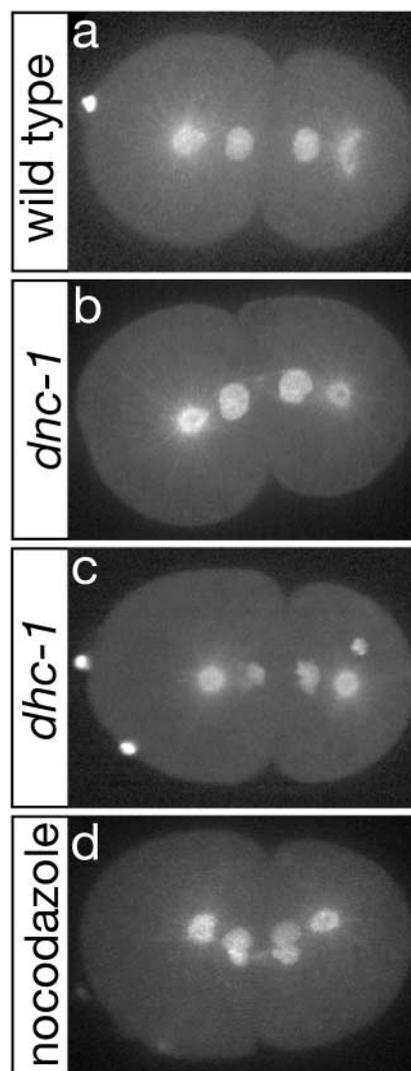


Figure 4. Dynein–dynactin function and intact MTs are required for centrosome flattening. (a) In wild-type embryos expressing tubulin–GFP and histone–GFP fusion proteins, the posterior centrosome becomes flattened in telophase. (b–d) Partial depletion of the dynactin component DNC-1 (b) or of the dynein heavy chain DHC-1 (c) disrupts centrosome flattening as does exposure to low doses of the MT-depolymerizing drug nocodazole (d).

to influence the shape and position of mitotic spindles. Both the dynactin complex and the minus end–directed MT motor dynein localize to the cortex of early embryos, and spindle rotation fails in two-cell stage embryos in which the dynein–dynactin complex has been partially depleted by RNA interference (Skop and White, 1998; Gönczy et al., 1999). Further reducing dynein–dynactin function disrupts pronuclear migration and the assembly and orientation of the first mitotic spindle (Gönczy et al., 1999). To determine whether dynein and dynactin are required for centrosome flattening in one-cell stage embryos, we partially depleted either the dynein heavy chain DHC-1 or a *C. elegans* orthologue of the dynactin component p150glued DNC-1 (see Materials and methods). The posterior centrosome remained spherical in all DNC-1-depleted embryos in which the first mitotic spindle rotated to lie along the a-p axis (Fig. 4 b; $n = 9$).

Similarly, we observed spherical centrosomes in some DHC-1-depleted embryos (Fig. 4 c; 4 out of 20 embryos; 4 embryos exhibited defects in chromosome segregation and in centrosome flattening, whereas 16 embryos appeared wild type during the first mitotic division). Exposure of embryos to low doses of nocodazole that shorten but do not eliminate MTs also disrupted centrosome flattening (Fig. 4 d; five out of seven embryos). We conclude that both dynein function and contact between astral MTs and the cortex are required for centrosome flattening.

Concluding remarks

Our data suggest that the nonmuscle myosin II subunits NMY-2 and MLC-4 mediate only a subset of F-actin-dependent processes during polarization of the a-p axis in a *C. elegans* zygote. F-actin is required for at least four polarity functions in the one-cell stage embryo: the cortical localizations of PAR-2, PAR-3, and NMY-2, and centrosome flattening (Fig. 5 a). In contrast, NMY-2 and MLC-4 are dispensable for cortical PAR localization and for centrosome flattening. Myosin II instead restricts PAR-3 to the anterior cortex, which permits expansion of the PAR-2 domain. As ectopic PAR-3 accumulates in the posterior of *par-2* single mutants, myosin is not sufficient to restrict PAR-3. Thus,

both PAR-2 and myosin II are required to limit PAR-3 to the anterior cortex.

The flattening of the posterior centrosome along the transverse axis may occur as a result of cortical forces that are applied to astral MTs and displace the first mitotic spindle toward the posterior pole (Grill et al., 2001; Tsou et al., 2002). The net magnitude of these forces is greater in the posterior hemisphere, and the posterior pole of the first mitotic spindle rocks from side to side during telophase. Thus, lateral forces act on astral MTs that contact the posterior cortex late in mitosis when centrosome flattening is observed. Because both spindle poles exhibit rocking motions in *par-3* mutant embryos, whereas neither pole shows rocking in *par-2* mutants, normal PAR polarity appears necessary to restrict lateral forces to the posterior pole (Cheng et al., 1995). Our data suggest that MFs recruit the dynein-dynactin complex to the cortex to apply these lateral forces to astral MTs. NMY-2 and MLC-4 are required for a polarized distribution of the PAR proteins, which in turn regulate the localization or the function of the dynein-dynactin motor complex, thus influencing both the position and shape of the first mitotic spindle (Fig. 5 b).

Recently, two models have been proposed to explain the establishment of asymmetry in the forces that position mitotic spindles in *C. elegans* zygotes. First, a DEP domain protein called LET-99 accumulates in a cortical stripe that is displaced toward the posterior pole, and high levels of LET-99 have been proposed to attenuate dynein-dependent forces applied to astral MTs that contact the cell cortex (Tsou et al., 2002). Properly positioned lateral attenuation would lower forces that normally oppose those applied to the spindle pole from the posterior-most cortex, producing a greater net force toward the posterior (see Fig. 7 in Tsou et al., 2002).

Alternatively, it has been suggested that MFs are unlikely to be involved in generating the cortical forces that act on spindle poles (Hill and Strome, 1988; Grill et al., 2001). This conclusion is based on experiments in which brief pulses of cytochalasin D, applied and washed out before anaphase, were sufficient to prevent posterior displacement of the first mitotic spindle during anaphase. Furthermore, cytochalasin D pulses applied during anaphase did not prevent posterior displacement (Hill and Strome, 1988). These findings suggest that MFs are not directly required for posterior displacement of the first mitotic spindle. Grill et al. (2001) therefore suggested that increased astral MT instability associated with the posterior cortex might account for the greater net posterior force. For example, such instability might facilitate pulling of the spindle pole toward the posterior cortex as astral MTs shorten.

Our findings support a role for MFs and the dynein-dynactin motor complex in applying forces to spindle poles via astral MTs that contact the cell cortex. It is possible that the pulses of cytochalasin D used by Hill and Strome (1988) were sufficient to disrupt some aspects of polarity but not to disrupt dynein-dynactin-mediated application of forces to astral MTs. Alternatively, cytochalasin D pulses may fully disrupt MF function, but two different force mechanisms could operate during spindle positioning. MT instability might account for posterior displacement, with dynein-dynactin forces generating only lateral rocking and flatten-

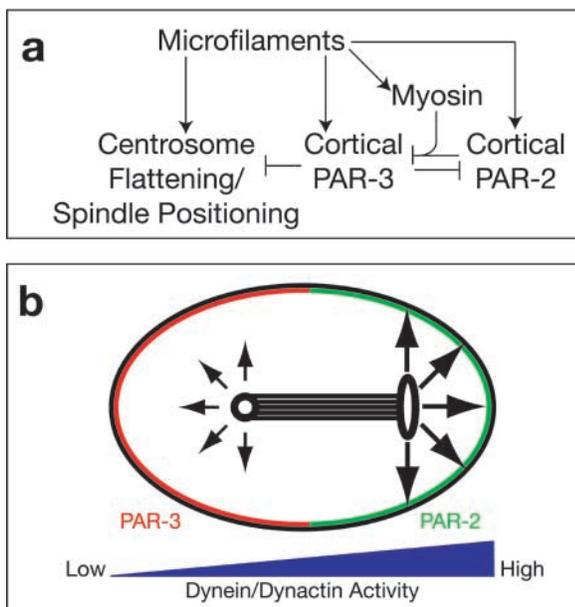


Figure 5. **Models of the polarization of the *C. elegans* zygote.**

(a) F-actin recruits myosin II, PAR-2, and PAR-3 to the cortex. In addition, MFs or MF-associated proteins act on the mitotic spindle, flattening the posterior centrosome. Myosin II and PAR-2 restrict PAR-3 to the anterior cortex where PAR-3 inhibits centrosome flattening. (b) A model of cortical forces that act in the early embryo. MFs recruit dynein and the dynactin complex to the cortex. Dynein pulls on astral MTs nucleated by the centrosomes. PAR-3 (red) inhibits dynein localization or function, resulting in a lower activity in the anterior hemisphere than in the posterior (blue triangle). Consequently, less force is applied to the anterior centrosome than the posterior centrosome (arrows), and the spindle becomes posteriorly displaced. The high lateral forces in the posterior hemisphere stretch the posterior centrosome, flattening it into a disc shape. For an alternative model see Tsou et al. (2002).

ing of the posterior spindle pole. In support of this possibility, we sometimes observed an absence of spindle pole flattening even though the spindle was displaced normally toward the posterior pole (Fig. 4). MF function is not limited to spindle flattening and rocking though, because MFs, dynein, and dynactin also are required for spindle rotation at the two-cell stage in wild-type and *par-3* mutant embryos. Finally, MT asters undergo abnormal lateral rocking movements early in mitosis in one-cell *let-99* mutant embryos, and this abnormal rocking also requires *dhc-1* (Tsou et al., 2002). We conclude that dynein–dynactin-mediated forces exert an extensive influence on mitotic spindle positioning in early *C. elegans* embryos.

Materials and methods

Strains and alleles

C. elegans strains were cultured as described previously; N2 Bristol was used as the wild-type strain (Brenner, 1974). The following alleles and balancer chromosomes were used in this study: LGIII: *par-2(lw32)*, *unc-45(e286ts)*, *lon-1(e185)*, *par-3(it71)*, *sC1[dpy-1(e1) let]*, and *qC1[dpy-19 glp-1]*.

Immunofluorescence and microscopy

Embryos were fixed and stained as described (Severson et al., 2000). Antibodies were diluted in PBS containing 3% BSA as follows: PAR-2, 1:20; PAR-3, 1:10; SPD-5, 1:1,000; and antiactin (ICN), 1:100. DNA was labeled with a 10-min incubation in 0.2 μ M TOTO-3 (Molecular Probes). Images were acquired using a Radiance laser-scanning confocal microscope (Bio-Rad Laboratories). For observations of centrosome shape following *dhc-1(RNAi)*, *dnc-1(RNAi)*, and nocodazole treatment, embryos expressing a histone–GFP and a β -tubulin–GFP fusion (Praitis et al., 2001) were mounted on a 4% agarose cushion and observed using a spinning disc confocal microscope (PerkinElmer).

LatA and nocodazole exposure

For LatA treatment, embryos were permeabilized by laser ablation of the eggshell (Severson et al., 2002) or by gentle pressure (Hill and Strome, 1988). Embryos were permeabilized during pronuclear migration for observations of centrosome flattening or after the completion of cytokinesis I for observations of spindle orientation in two-cell embryos. Embryos were incubated for at least 10 min in culture medium containing 100 μ M LatA (prepared from a 10 mM stock in DMSO) or in DMSO as a control and then observed by Nomarski microscopy or fixed and processed for immunocytochemistry. Centrosome flattening, spindle orientation, and PAR localization were normal in DMSO-treated embryos, and cell cycle progression continued normally in both LatA- and DMSO-treated embryos. For nocodazole treatment, prepronuclear migration stage embryos were mounted on a 4% agarose cushion under a coverslip and then bathed in 20 μ g/ml nocodazole in M9 prepared from a 1 mg/ml stock solution in DMSO.

RNAi

Double stranded RNA was prepared and injected by standard methods (Fire et al., 1998). The following cDNA clones were used as templates: *mhc-4*, yk167f10; *nmy-2*, yk45d7; and *pfn-1*, yk402e3. Sequences corresponding to *dhc-1* and *dnc-1* were amplified from genomic DNA using the following primers: *dhc-1*: aaggaaggagctcaacgaca, ccttctctctgggtcttc; and *dnc-1*: tcacgaatcctcggtttc, gaagcagcgggtgattat. PCR products were cloned into PCRII-TOPO (Invitrogen), and single stranded RNA was transcribed with T7 polymerase, injected at a concentration of 1 mg/ml, and embryos were analyzed 18–20 h postinjection.

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