The forces that position a mitotic spindle asymmetrically are tethered until after the time of spindle assembly

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Regulation of the mitotic spindle's position is important for cells to divide asymmetrically. Here, we use *Caenorhabditis elegans* embryos to provide the first analysis of the temporal regulation of forces that asymmetrically position a mitotic spindle. We find that asymmetric pulling forces, regulated by cortical PAR proteins, begin to act as early as prophase and prometaphase, even before the spindle forms and shifts to a posterior position. The spindle does not shift asymmetrically during these early phases due to a tethering force, mediated by astral microtubules that reach the anterior cell cortex. We show that this tether is normally released after spindle assembly and independently of anaphase entry. Monitoring microtubule dynamics by photobleaching segments of microtubules during anaphase revealed that spindle microtubules do not undergo significant poleward flux in *C. elegans*. Together with the known absence of anaphase A, these data suggest that the major forces contributing to chromosome separation during anaphase originate outside the spindle. We propose that the forces positioning the mitotic spindle asymmetrically are tethered until after the time of spindle assembly and that these same forces are used later to drive chromosome segregation at anaphase.

Introduction

Asymmetric positioning of the mitotic spindle is important for unequal cell divisions to occur during embryonic development and in stem cells (Kaltschmidt and Brand, 2002). Mitotic spindle positioning is known to depend on microtubule–cortical interactions in several animal systems (Lutz et al., 1988; Hyman, 1989; Goldstein, 1995; Yamashita et al., 2003). We are making use of the *Caenorhabditis elegans* embryo to understand how asymmetrically activated or localized proteins can result in the production of forces that can drive movement of the mitotic spindle to one side of an animal cell.

The first division of the early *C. elegans* embryo gives rise to two daughters of different size and molecular composition; e.g., only the posterior daughter inherits germline determinants such as P granules and the protein PIE-1 (Pellettieri and Seydoux, 2002). This asymmetry in cell size results from the position of the first mitotic spindle, which forms at the center but moves to the posterior of the one-cell embryo before cytokinesis (Albertson, 1984). Posterior spindle displacement is dependent on the PAR proteins, which are required to establish and maintain polarity

in the embryo (Kemphues et al., 1988), and heterotrimeric G protein signaling, which acts downstream of the PAR proteins (Gotta and Ahringer, 2001). Spindle-cutting experiments demonstrated that PAR proteins and G proteins function to generate an imbalance in pulling forces that act on each side of the spindle during anaphase, creating a stronger pulling force toward the posterior of the embryo and possibly regulating posterior spindle displacement (Grill et al., 2001, 2003). These experiments also demonstrated that spindle microtubules function to limit the rate of spindle pole separation during anaphase (Grill et al., 2001), possibly because the antiparallel sliding of polar microtubules in the spindle occurs at a limiting rate.

Interestingly, both the PAR protein and G protein pathways have been shown to be conserved in *Drosophila melanogaster*, where they are required to establish oocyte polarity and regulate the asymmetric division of neuroblasts (for reviews see Knoblich, 2001; Pellettieri and Seydoux, 2002). PAR proteins are also localized at tight junctions in mammalian epithelial cells, where they regulate various aspects of cell polarity (Izumi et al., 1998; Joberty et al., 2000; Hurd et al., 2003). These findings suggest that mechanisms of cell polarization studied in *C. elegans* may be widely used in polarized animal cells.

Although little is known about how the mitotic spindle is positioned asymmetrically before asymmetric cell divisions, there has been intensive study on the generation of forces that drive movements of spindle components during normal mitotic

divisions. Such work is informative, and also provides model approaches, for studying how spindles are positioned asymmetrically. Segregation of chromosomes, for example, occurs through a fine regulation of microtubule-dependent forces that act on centrosomes and sister chromatids through the mitotic phase of the cell cycle (for review see Cleveland et al., 2003). These forces have been defined as anaphase A and B forces.

Anaphase A forces function to shorten the distance between each sister chromatid and its respective spindle pole. In *Drosophila* embryos and *Xenopus laevis* extract spindles, these forces are mediated, at least in part, by a complex regulation of microtubule dynamics: the kinetochore microtubules, which directly mediate the connection between chromosomes and the spindle pole, generally undergo a process termed poleward flux, a microtubule behavior in which the kinetochore-bound plus end of microtubules undergoes polymerization, whereas the spindle pole-associated minus end is concomitantly depolymerized (Mitchison, 1989; Desai et al., 1998; Brust-Mascher and Scholey, 2002; Maddox et al., 2002). At metaphase, the rates of microtubule polymerization and depolymerization are equal, and individual tubulin dimers translocate along microtubules in a plus-to-minus end direction, leaving kinetochore microtubules at a roughly constant length (Mitchison, 1989; Maddox et al., 2003). At anaphase onset, the microtubule plus ends switch from polymerization to depolymerization, whereas the minus ends continue to depolymerize, resulting in movement of the chromosomes toward the spindle pole (Desai et al., 1998; Maddox et al., 2003).

Anaphase B forces are responsible for the increase in distance between the two spindle poles, which generally occurs at anaphase onset. This increase can occur through the generation of pushing forces by motors on overlapping, antiparallel spindle microtubules (Aist and Berns, 1981; Inoue et al., 1998). Pole–pole separation can also be mediated by astral microtubules, which extend from the centrosomes and make contact with the cell cortex. For instance, cortically bound, minus end–directed motor proteins, such as dynein, could mediate such a function (Inoue et al., 1998). Astral microtubules are also required to position the spindle in the center of dividing cells (O'Connell and Wang, 2000). In symmetrically dividing cells, the forces acting on astral microtubules are likely equal on each side of the spindle and remain equal during both spindle positioning and spindle pole separation.

Both anaphase A and anaphase B forces are temporally regulated by the cell cycle machinery. This level of regulation is mediated, in part, by components of the spindle checkpoint and ensures that segregation does not initiate before all chromosomes make kinetochore–microtubule attachments and align at the metaphase plate (for review see Cleveland et al., 2003). Interestingly, during prometaphase and metaphase, poleward microtubule flux as well as microtubule plus end dynamics generate forces, as evidenced by tension at the kinetochore (Pearson et al., 2001). These forces contribute to chromosome congression and are at dynamic equilibrium when sister chromatids are aligned at the metaphase plate, indicating that some forces are active before cells enter anaphase (Mitchison and Salmon, 1992; Waters et al., 1996). The absence of chromo-some segregation during this time might be mediated by cohesins, which link sister chromatids together and are degraded at the metaphase–anaphase transition (Nasmyth, 2002).

As a step toward understanding how pulling forces are regulated to achieve posterior spindle displacement and chromosome segregation in asymmetrically dividing cells, we set out to determine how an imbalance in pulling forces arises during progression through the cell cycle. We have used laser disruption of centrosomes and measurement of movement of unirradiated centrosomes to produce a map of the forces acting on centrosomes throughout M phase of an asymmetric division. We find that asymmetric pulling forces normally begin to act on the mitotic spindle as early as prophase and prometaphase—before the time of posterior spindle displacement. Astral microtubules that contact the anterior cortex contribute a tethering force that restrains movement of the spindle during late prophase and prometaphase. We show that this anterior tether is normally released before anaphase entry and independently of the metaphase to anaphase transition and that these forces are controlled by the cortically enriched PAR proteins. Photobleaching segments of microtubules during anaphase revealed that spindle microtubules are not undergoing significant poleward flux. Together with the known absence of anaphase A, these data suggest that forces from outside the spindle are the major components contributing to chromosome separation during anaphase. We propose that the forces acting on microtubules to asymmetrically position the mitotic spindle are modulated throughout the cell cycle and that these same forces are used to drive chromosome segregation at anaphase. These results provide the first analysis of the temporal regulation of spindle-positioning forces in an asymmetrically dividing animal cell.

Results

Asymmetric spindle positioning begins in metaphase

In the one-cell stage *C. elegans* embryo, the spindle forms at the center of the cell and moves toward the posterior before cytokinesis. Previous experiments assessing the forces acting on the spindle were performed at anaphase B (Grill et al., 2001, 2003). As a baseline for further studies, we first determined the stage of the cell cycle during which posterior spindle displacement occurs by imaging embryos expressing both γ-tubulin and histone H2B fused to GFP (Oegema et al., 2001), which allowed us to simultaneously monitor the behavior of centrosomes and chromosomes, respectively. After the spindle arrived at the center of the embryo, both the centrosomes and chromosomes began to move posterior of the center 60.9 ± 20.8 s before anaphase ($n = 7$), near the time when sister chromatids were first aligned at the metaphase plate (Fig. 1 and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200406008/DC1). Chromosome separation occurred after the spindle began moving toward the posterior in the cell, and the posterior spindle pole continued to move posteriorly after entry into anaphase. Therefore, the mitotic spindle begins to move to an asymmetric position during metaphase, before anaphase on-
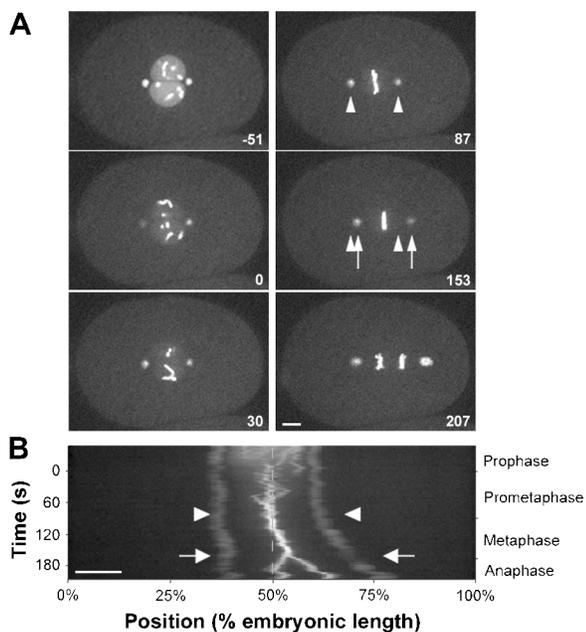
Figure 1. **Posterior spindle displacement begins at metaphase.** (A) Time-lapse images of an early *C. elegans* embryo expressing both γ-tubulin and histone H2B fused to GFP. (B) Kymograph analysis of spindle behavior from these time-lapse images. In both panels, arrowheads point to centrosomes at early metaphase and arrows point to the centrosomes at late metaphase, before anaphase onset. Displacement of the spindle toward the posterior can be observed during metaphase. Displacement began during early metaphase or at the end of prometaphase in all embryos examined in this way (n = 8; see Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200406008/DC1). Bars, 5 μm.

set, which is consistent with observations made previously (Oegema et al., 2001). This result suggests that spindle positioning is unlikely to be regulated by anaphase entry.

To determine whether or not anaphase entry is indeed dispensable for posterior spindle displacement, we examined if posterior spindle displacement can occur when cells are arrested in metaphase. In eukaryotic cells, the transition from metaphase to anaphase depends on the activity of the proteasome, which degrades proteins such as securin and B-type cyclins (for reviews see Nasmyth, 2002; Peters, 2002). Spindle positioning was monitored in embryos treated with *clasto*-lactacystin β-lactone (c-LβL), a potent, irreversible inhibitor of the 26S proteasome (see Materials and methods). The initial timing and progression of the first cell cycle events in c-LβL–treated embryos was normal, and embryos entered mitosis at the normal time (Fig. 2 A). As expected, the treated embryos failed to complete mitosis. Monitoring centrosome separation revealed that these embryos were arrested in metaphase and did not undergo anaphase B (Fig. 2 B). Such c-LβL–treated embryos could remain in this arrested state for extended periods of time (at least 30 min) and remained arrested even when pole separation was allowed to occur (see online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200406008/DC1).
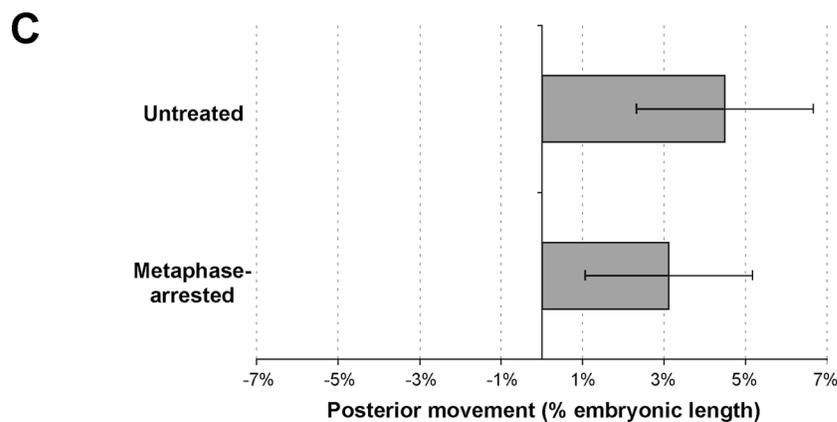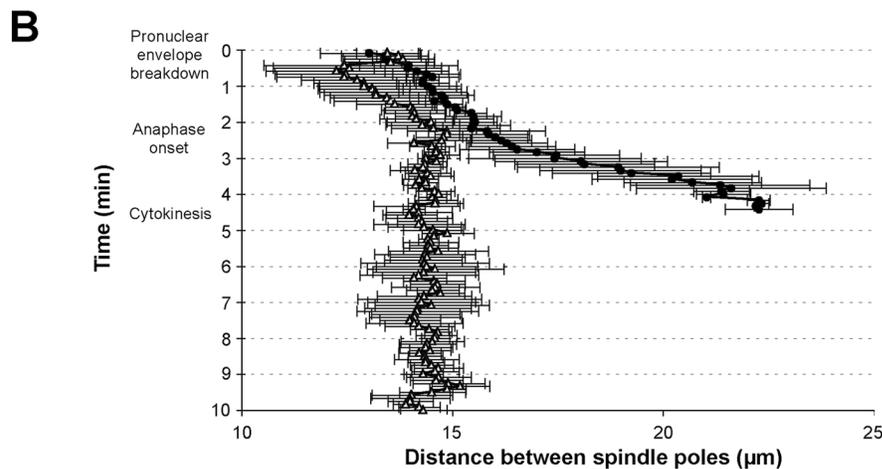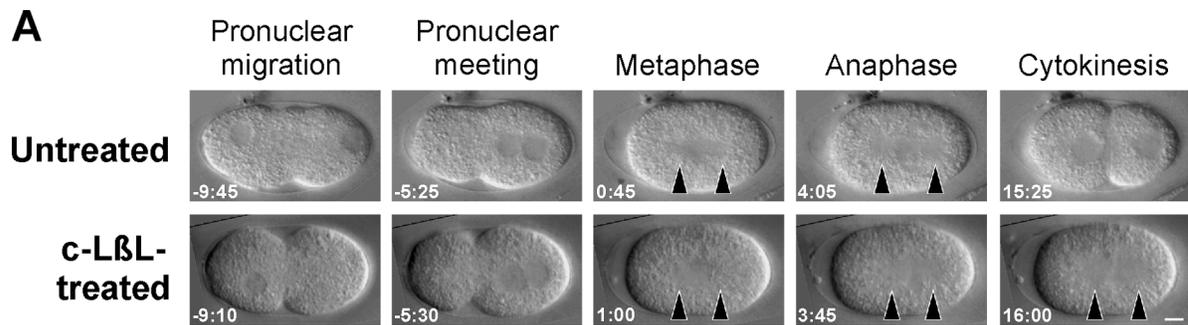
We monitored spindle positioning in such metaphase-arrested embryos and observed that posterior spindle displacement occurred normally. Indeed, posterior spindle displacement started at the correct time and proceeded in a manner

similar to that in untreated embryos (Fig. 2 A). Furthermore, the extent of posterior spindle displacement at the time of anaphase onset was indistinguishable from that in untreated embryos (4.5 ± 2.2% embryonic length [EL] compared with 3.1 ± 2.1% EL, n = 5 and n = 4 for spindle displacement in untreated and c-LβL–treated embryos, respectively, P = 0.36; Fig. 2 C). This result demonstrates that the onset of posterior spindle displacement does not depend on anaphase entry.

An asymmetry in pulling forces exists before the spindle starts moving toward the posterior

To characterize the forces that drive posterior spindle displacement, we examined in detail the regulation of spindle-positioning pulling forces through the first cell cycle of the *C. elegans* embryo. Previous experiments have used spindle severing to reveal that spindle poles are pulled apart during anaphase B (Grill et al., 2001), indicating that pulling forces are acting on spindle poles during this stage of the cell cycle. To examine the forces when posterior spindle displacement begins and also throughout the cell cycle, we used an approach termed optically induced centrosome disruption (OICD; Grill et al., 2003), in which we laser irradiated either the anterior or posterior centrosome during various phases of the cell cycle and monitored the resulting movement of the nonirradiated centrosome (see Materials and methods). Our OICD experiments conducted during anaphase succeeded in replicating previous spindle-cutting results (Grill et al., 2001). We found that the OICD procedure was efficient in disrupting one of the two asters without detectably affecting the organization of the nonirradiated one (Fig. 3 A and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200406008/DC1). After irradiation of one of the two centrosomes, the spindle was left with only one of the asters maintaining extensive connection with the cell cortex. Quantification of centrosome movement was used to estimate the net relative vectorial force applied on this aster (Fig. S3 and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200406008/DC1). Using this approach, we generated a map of relative pulling forces acting on each aster throughout the cell cycle (Fig. 3 B). Because the timing of rotation is variable during centration in wild-type embryos, centrosome irradiations that were performed during rotation were performed in the embryos that had undergone a minimum of 45° rotation before having reached half the distance to the center of the embryo.

In wild-type one-cell embryos, at least two microtubule-dependent movements are apparent during early development. One of these two movements occurs during early prophase, when the two pronuclei and their associated centrosomes migrate from the posterior to the center of the embryo (Albertson, 1984; Hyman and White, 1987). Irradiating the centrosomes during this time showed that the anterior centrosome is subjected to a greater net force toward the anterior than the posterior centrosome toward the posterior (Fig. 3 B). After OICD, movement of the anterior centrosome toward the anterior of the embryo was about 10 times greater than movement of the posterior centrosome toward the posterior of the embryo (11.0 ±

A

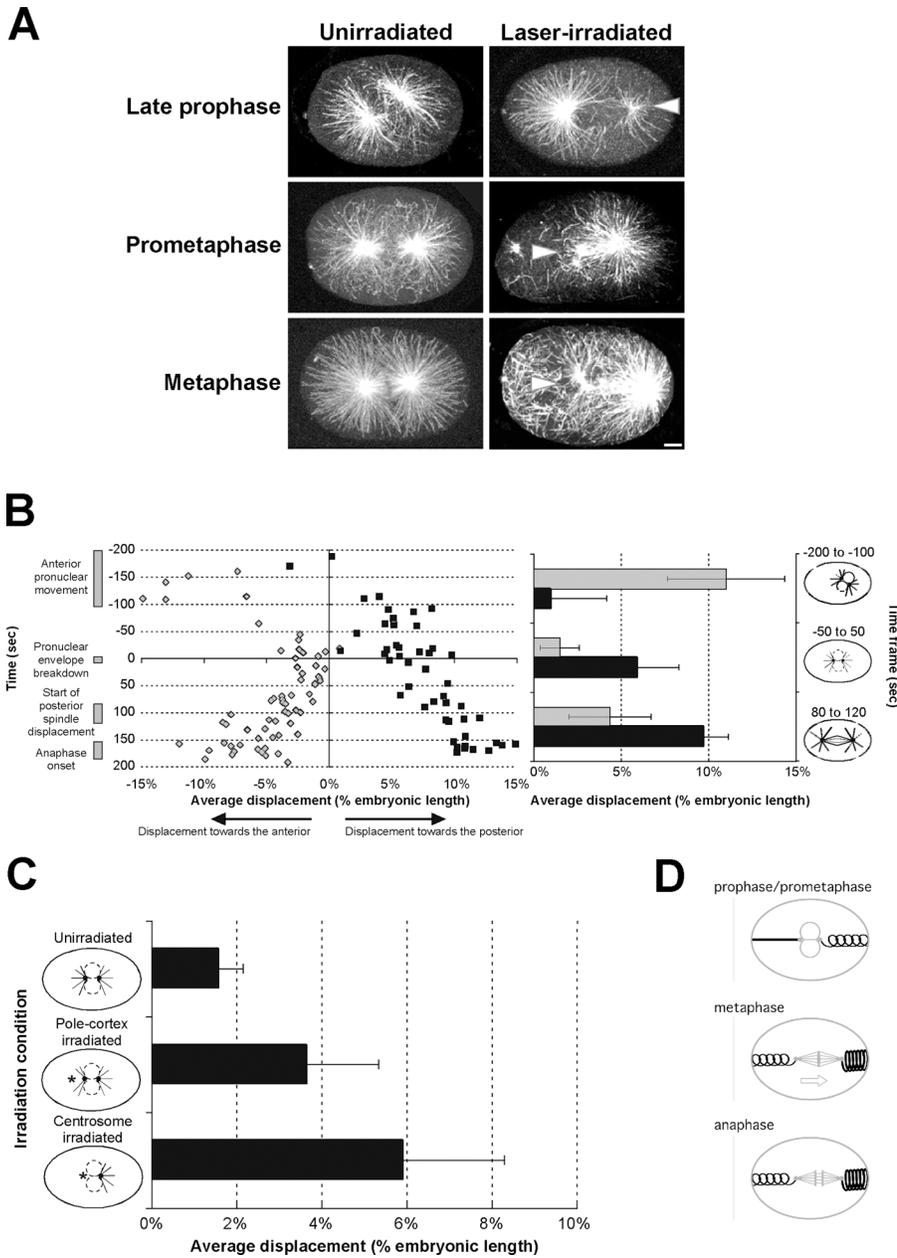|	Pronuclear migration	Pronuclear meeting	Metaphase	Anaphase	Cytokinesis
Untreated	-9:45	-5:25	0:45	4:05	15:25
c-LßL-treated	-9:10	-5:30	1:00	3:45	16:00

B

C

Figure 2. **Posterior spindle displacement occurs in metaphase-arrested embryos.** (A) Time-lapse images of untreated wild-type embryos and wild-type embryos treated with c-LβL during pronuclear migration, before meeting. All cellular events appear to occur normally in both cases until anaphase onset. Embryos treated with c-LβL remain arrested in metaphase and fail to undergo cytokinesis. Arrowheads indicate the position of anterior and posterior centrosomes in these time-lapse images. Time is indicated in minutes and the 0 time point is at pronuclear envelope breakdown. Bar, 5 μm. (B) Quantification of the distance between the two spindle poles in untreated (closed circles) and c-LβL–treated (open triangles) wild-type embryos. At the normal time of anaphase onset, the spindle fails to elongate in treated embryos and remains at constant length for at least 30 min. The 0 time point corresponds to pronuclear envelope breakdown. (C) Quantification of the extent of posterior spindle displacement at anaphase onset in untreated and c-LβL–treated, metaphase-arrested wild-type embryos. The spindle initiates posterior movement at the correct time and moves a comparable distance in both cases, and the spindle midpoint was positioned at 56 ± 2% EL in untreated embryos and 56 ± 3% EL in c-LβL–treated embryos. In all panels, n = 5 for untreated embryos and n = 4 for c-LβL–treated embryos. Error bars represent SD over five and four embryos.

3.5% EL compared with 1.0 ± 3.2% EL, n = 6 and n = 4 for anterior and posterior centrosome movement, respectively, P = 0.001). This result is consistent with the observed net movement of the pronuclei–centrosomes complex toward the anterior during this time.

Another microtubule-dependent movement occurs during metaphase, when the mitotic spindle begins to move toward the posterior of the embryo (Albertson, 1984; Kemphues et al., 1988). Irradiation of the anterior or posterior aster during this time of the cell cycle demonstrated that a stronger net pulling force is present on the posterior aster compared with the anterior aster (Fig. 3 B). At the onset of posterior spindle displacement, during metaphase, movement of the posterior centrosome toward the posterior of the embryo was about twice as

A

	Unirradiated	Laser-irradiated
Late prophase		
Prometaphase		
Metaphase		

B

C

D

prophase/prometaphase

metaphase

anaphase

Figure 3. **Laser-mediated disruption of microtubule organization allows the estimation of pulling forces throughout the cell cycle.** (A) Two-dimensional reconstruction of multiple confocal sections of embryos fixed and stained for α-tubulin. The centrosomes indicated by arrowheads in the right panels were irradiated before fixation. Laser irradiation specifically disrupts microtubule organization at the targeted centrosome, leaving the unirradiated centrosome largely intact. Bar, 5 μm. (B) Map (left) and quantification (right) of centrosome displacement after OICD. Displacement was determined for anterior centrosomes after posterior centrosome irradiation (gray diamonds and gray bars) and for posterior centrosomes after anterior centrosome irradiation (black squares and black bars). In the right panel, displacements were averaged for various phases of the cell cycle. The timing of cell cycle events was determined according to previously reported values and matches observations made by DIC optics (Labbé et al., 2003). Error bars represent SD over, from top to bottom, 6, 4, 19, 16, 13, and 8 embryos, respectively, for each case. (C) Posterior centrosome movement quantified following no irradiation, laser irradiation of a region between the anterior centrosome and the anterior cortex, or laser irradiation of the whole anterior centrosome. A schematic of the procedure for each condition is shown on the left, with the region irradiated marked with an asterisk. Error bars represent SD over, from top to bottom, 8, 8, and 16 embryos, respectively, for each case. (D) Conceptual model depicting the various types of forces that act on the centrosomes throughout the first mitosis of the early *C. elegans* embryo. In this model, tethering forces are represented as lines, whereas pulling forces are depicted as springs. At late prophase/prometaphase, the pulling force present in the posterior of the embryo is counteracted by the tethering force in the anterior, thereby preventing posterior spindle displacement. During metaphase, the tethering force in the anterior changes to a pulling force.

great as the movement of the anterior centrosome toward the anterior of the embryo (4.4 ± 2.3% EL compared with 9.7 ± 1.4% EL, $n = 13$ and $n = 8$ for anterior and posterior centrosome movement, respectively, P = 1.3E−5). A comparable result has been found during anaphase, when a 40% difference exists in centrosome peak velocities between anterior and posterior centrosomes, after central spindle cutting during anaphase, when transverse spindle movement was observed (Grill et al., 2001). These results recapitulate the known microtubule-dependent forces and movements that occur during early prophase and metaphase, and therefore suggest that our OICD approach is valid to study relative forces during the first embryonic cell cycle.

A surprising result was obtained when performing OICD experiments around the time of pronuclear envelope breakdown, during late prophase and prometaphase. Laser irradiation of the anterior or posterior aster during this time demonstrated that an asymmetry in pulling forces is already present before the start of posterior spindle displacement: during late prophase and prometaphase, irradiation of the anterior centrosome resulted in posterior displacement of the posterior centrosome, whereas irradiating the posterior centrosome at this time caused little anterior movement of the anterior centrosome (Fig. 3 B). After irradiation at this stage, the movement of the posterior centrosome toward the posterior of the embryo was four times greater than movement of the anterior centrosome toward the anterior (1.5 ± 1.1% EL compared with 5.9 ± 2.4% EL, $n = 19$ and $n = 16$ for anterior and posterior centrosome movement, respectively, P = 1.6E−8). This finding indicated that, during late prophase/prometaphase, the posterior aster is being pulled by a stronger net force than the anterior one, and therefore that an asymmetry in pulling forces exists before posterior spindle displacement.

A microtubule-based tether is released near the time of the metaphase–anaphase transition

During late prophase and prometaphase, the centrosomes move very little toward the posterior of the embryo (Fig. 1 and Fig. 3 C), indicating that forces are roughly equal on each side of the spindle. However, our OICD results indicated that an asymmetry in pulling forces is present at these stages—a pulling force is active on the posterior centrosome, whereas little pulling force is detectable on the anterior centrosome. Furthermore, the relatively weak net pulling force estimated on the anterior aster during late prophase/prometaphase suggested that the anterior centrosome is not actively being pulled during this time. Although a small amount of active pulling force toward the anterior was detected, this relatively weak force, as well as the absence of significant spindle movement in untreated embryos despite strong pulling on the posterior aster, suggested to us that microtubules from the anterior aster tether the spindle at the anterior cortex during late prophase and prometaphase. Tethering of microtubules to the anterior cortex might account for the force that counterbalances the posterior pulling force that we observed, thereby maintaining the net forces at equilibrium and keeping the centrosomes near the center of the embryo.

To test this hypothesis, we laser irradiated cytoplasmic regions between the centrosome and the cortex, near the centrosome (Fig. 3 C). We found that we could selectively deplete populations of astral microtubules from various regions around the centrosome, leaving the unirradiated regions largely intact, as done previously (Hyman, 1989; unpublished data). If anterior microtubules are responsible for generating a tethering force, then irradiating between the anterior centrosome and the anterior cortex should release this tether and trigger movement of the posterior centrosome toward the posterior. This is what we observed: whereas the posterior centrosome normally moves $1.6 \pm 0.6\%$ EL ($n = 8$) during late prophase/prometaphase, this movement increases to $3.7 \pm 1.7\%$ EL ($n = 8$) toward the posterior after irradiation between the anterior aster and the cortex, a difference that is statistically significant (P = 0.005; Fig. 3 C). The displacement of the posterior centrosome after irradiation between the anterior aster and the cortex is not as large as the displacement of the posterior centrosome after the complete disruption of the anterior aster ($3.7 \pm 1.7\%$ EL compared with $5.9 \pm 2.4\%$ EL, $n = 8$ and $n = 16$, respectively), probably due to the fact that more microtubules from the anterior aster are able to mediate contact with the lateral cortex when the aster is not as completely disrupted. Irradiating between the posterior centrosome and the posterior cortex during late prophase/prometaphase resulted in very little movement of the anterior centrosome ($0.2 \pm 0.1\%$ EL, $n = 14$). These results are consistent with our proposal that the anterior microtubules are tethering the pronuclear–centrosome complex to the anterior cortex during this stage, thereby counteracting the effect of the posterior pulling force and allowing the complex to remain at the center of the embryo.

Comparison of centrosome movement after OICD at late prophase/prometaphase, metaphase, and anaphase demonstrated that a change in forces occurs at the anterior during cell cycle progression; after irradiation of the posterior aster during metaphase, the movement of the anterior centrosome toward the anterior of the embryo was three times greater than after irradiation of the posterior aster during late prophase/prometaphase ($4.4 \pm 2.3\%$ EL compared with $1.5 \pm 1.1\%$ EL, $n = 13$ and $n = 19$, respectively, P = 6.8E−5; Fig. 3 B). Following irradiation during anaphase, the extent of movement of the posterior centrosome toward the posterior of the embryo was higher than movement of the anterior centrosome toward the anterior of the embryo ($6.8 \pm 2.5\%$ compared with $11.9 \pm 1.6\%$, $n = 12$ each for anterior and posterior centrosome movement, respectively, P = 4.9E−6), which is consistent with that reported by Grill et al. (2001). The finding that targeting centrosomes produces a result indistinguishable from previous cutting of spindles further suggests that targeting one centrosome does not significantly alter forces at the other centrosome. Together, these results indicate that the forces on the anterior aster undergo a transition, from tethering to active pulling, between pronuclear envelope breakdown and the start of posterior spindle displacement (Fig. 3 D).

The transition in forces occurs independently of the metaphase–anaphase transition

That forces transition from tethering to pulling in the anterior of the embryo during mitosis prompted us to ask whether or not the forces are regulated by cell cycle transitions. To test this, we irradiated the posterior centrosome in c-LβL–treated embryos at various times during the cell cycle. If forces are not regulated by the cell cycle machinery, then the anterior pulling force observed at the onset of posterior spindle displacement in metaphase-arrested embryos should transition to the stronger force, comparable to the net anterior force observed at metaphase in untreated embryos. We performed laser irradiations of centrosomes in metaphase-arrested embryos and confirmed this hypothesis; when the posterior centrosome was irradiated at the time of
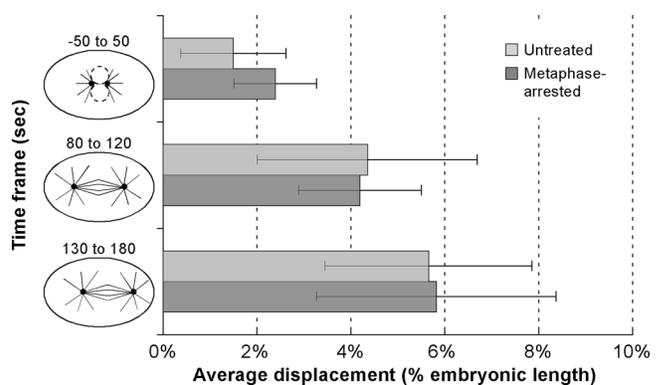

Figure 4. **A transition in forces occurs in metaphase-arrested embryos.** Quantification of anterior centrosome movement after posterior centrosome irradiation in untreated (light gray bars) and c-LβL–treated, metaphase-arrested wild-type embryos (dark gray bars). Displacement of the anterior centrosome toward the anterior increases as the cell cycle progresses from late prophase/prometaphase to metaphase. Error bars represent SD over, from top to bottom, 19, 5, 13, 8, 8, and 9 embryos, respectively, for each case.

metaphase in c-LβL–treated embryos, we observed a net anterior movement of the anterior centrosome similar to the net anterior movement observed at the onset of posterior spindle displacement in untreated embryos (4.2 ± 1.3% EL compared with 4.4 ± 2.3% EL, $n = 8$ and $n = 13$, respectively, P = 0.89; Fig. 4). Irradiating the posterior centrosome at the time of late prophase, with or without treatment with the proteasome inhibitor, gave indistinguishable results (Fig. 4), thereby indicating that forces are not grossly misregulated in c-LβL–treated embryos. This result suggests that the pulling forces responsible for moving the spindle posteriorly are not regulated by the proteasome-dependent events at the metaphase–anaphase transition during progression through mitosis in the early embryo.

Spindle-positioning forces also drive sister chromatid segregation

In vertebrate and *Drosophila* spindles, microtubule poleward flux is a significant component of chromosome segregation (Desai et al., 1998; Maddox et al., 2002, 2003). One striking observation made previously in *C. elegans* embryos is that the mitotic spindle does not undergo anaphase A during chromo-some segregation (Oegema et al., 2001; Fig. 5, A and E). Furthermore, despite an asymmetry in pulling forces on each side of the spindle, we have found that chromosome segregation and centrosome separation in each spindle half appear symmetric (Fig. 5 A), suggesting that the forces within the mitotic spindle itself may also be symmetric. Together with the finding that the spindle midzone limits the rate of anaphase pole separation (Grill et al., 2001), these observations suggested that the forces responsible for mediating pulling on the asters during posterior spindle displacement may also drive the segregation of chromosomes at anaphase B. However, poleward flux has been shown to generate tension at the kinetochore of mitotic spindles in *Xenopus* extracts, through constant microtubule plus end net polymerization and minus end depolymerization (Desai et al., 1998; Maddox et al., 2003). Therefore, one possibility remained that spindle microtubules might be under tension during chromosome segregation through poleward flux, despite the apparent absence of anaphase A.

To test this possibility, we used an approach that relies on the photobleaching of microtubule-associated fluorophores and quantification of FRAP in living specimens. Such an approach
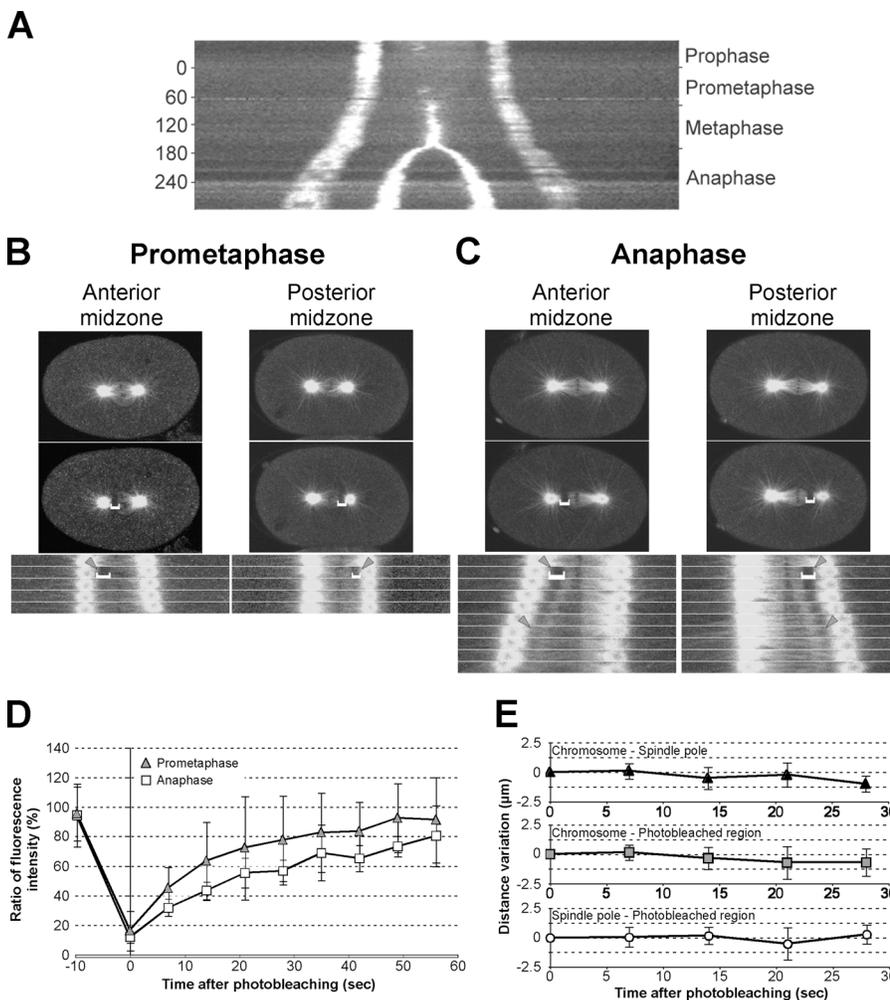
Figure 5. **Chromosome segregation occurs without anaphase A and significant poleward flux.** (A) Spindle-centered kymograph of an embryo expressing both γ-tubulin and histone H2B fused to GFP. This kymograph was produced similarly to the one in Fig. 1 B, except that posterior movement of the spindle was eliminated: the spindle in each frame of time-lapse images was rotationally aligned and recentered on the midpoint between the centrosomes to allow the observation of symmetries in the spindle. Frames were acquired at 7-s intervals. (B and C) Time-lapse images of embryos expressing β-tubulin::GFP in which a short region of anterior (left) or posterior (right) spindle microtubules were photobleached during prometaphase (B) or anaphase (C) onset. The bottom panels follow FRAP as well as movement of the photobleached region (indicated by gray arrowheads). Frames were acquired at 7-s intervals. For photobleaches of the spindle during prometaphase, kymographs were aligned to the location of chromosomes in the center of the spindle. For photobleaches of the anterior or posterior half of the spindle at anaphase onset, kymographs were aligned to the center of the posterior or anterior centrosome, respectively. (D) Quantification of FRAP during prometaphase (gray triangles) and anaphase (open squares). To correct for fluorophore bleaching and embryo to embryo variations, fluorescence intensity in the photobleached region is expressed as a ratio of bleached over unbleached midzone microtubules in the same embryo. FRAP occurs faster during prometaphase ($t_{1/2} = 10.6$ s; polynomial equation: $y = -3E - 07 x^4 + 4E - 05x^3 - 0.0021x^2 + 0.0559x + 0.1571$; $R^2 = 0.997$) compared with anaphase ($t_{1/2} = 17.7$ s; polynomial equation: $y = 4E - 08x^4 + 2E - 06x^3 - 0.0005x^2 + 0.0303x + 0.1223$; $R^2 = 0.989$). Time points were acquired at 7-s intervals. Error bars represent SD over six embryos. (E) Quantification of the distance variation between chromosomes to photobleached region (black triangles), chromosomes and spindle poles (gray squares), and spindle pole to photobleached region (open circles) during anaphase. The distance remains constant between these three positions throughout anaphase. Time points were acquired at 7-s intervals. Error bars represent SD over six embryos.

has proven successful in the past to study a broad variety of cellular events, including the dynamics of spindle microtubules (Salmon et al., 1984; Saxton et al., 1984; Zhai et al., 1995). We photobleached a small region of the central spindle in embryos expressing a gene encoding β-tubulin fused to GFP at either prometaphase or at anaphase onset and quantified FRAP in this region (see Materials and methods). Spindle microtubules photobleached at the time of prometaphase showed a fast fluorescence recovery time (average $t_{1/2} = 10.6$ s), suggesting a rapid turnover of tubulin subunits in the microtubule polymer during this stage of the cell cycle (Fig. 5, B and D). This fast recovery precluded detecting if the photobleached region moved during metaphase, thus preventing analysis of microtubule dynamic properties, such as poleward flux, because no mark could be followed on the microtubule lattice. However, microtubules photobleached at anaphase onset showed a slower rate of fluorescence recovery (average $t_{1/2} = 17.7$ s), indicating a slower turnover of tubulin subunits within microtubules at this stage (Fig. 5, C and D; and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200406008/DC1). We were able to monitor the movement of the photobleached region during the course of anaphase and found that the photobleached region on spindle microtubules remained at a constant distance from the spindle pole as it followed the spindle pole (Fig. 5 E). This finding demonstrates that, during anaphase, spindle microtubules are not undergoing significant poleward flux in *C. elegans* embryos. The fact that a majority of spindle microtubules are mediating kinetochore attachments in *C. elegans* (O'Toole et al., 2003) suggests that a significant number of the bleached microtubules are attached to kinetochores. The finding that kinetochore microtubules do not undergo significant flux implies that kinetochore microtubules do not contribute dynamic forces during anaphase. Together with the findings that the spindle midzone limits the rate of anaphase pole separation (Grill et al., 2001) and the absence of anaphase A (Oegema et al., 2001), these results suggest that in *C. elegans* spindle microtubules are relatively static in anaphase. We conclude that the forces that drive pole and chromosome separation are provided by astral microtubules.

PAR proteins regulate asymmetric pulling forces throughout the mitotic phase of the cell cycle

In *C. elegans* embryos, cortically localized PAR proteins function to establish and maintain polarity, and their disruption results in a failure in posterior spindle displacement (Kemphues and Strome, 1997). Therefore, we asked whether or not the posterior pulling force that we observed during late prophase and prometaphase was under the regulation of the *par* genes. The asymmetry in pulling forces observed at a later stage, during anaphase B, was previously reported to depend on the activity of the *par-2* and *par-3* genes (Grill et al., 2001). We sought to test whether or not these genes regulate forces throughout the cell cycle, and specifically as spindle displacement began.

We performed OICD in *par-2(lw32)* or *par-3(it71)* mutant embryos and found that the pulling forces that are acting on anterior and posterior asters during late prophase/

prometaphase are more similar to each other than in wild-type embryos (Fig. 6). The estimated forces on anterior and posterior asters in both *par-2* and *par-3* mutants during late prophase and prometaphase were roughly halfway between those estimated on anterior and posterior asters in wild-type embryos; the overall displacement in *par-2* mutant embryos was different from wild-type anterior as well as from wild-type posterior centrosomes, and the same was observed when comparing displacement in *par-3* mutant embryos with that of wild-type anterior and wild-type posterior centrosomes (Fig. 6 legend, numbers). However, the difference in overall displacement between *par-2* and *par-3* mutant embryos was not statistically significant ($3.0 \pm 2.1\%$ EL compared with $3.4 \pm 1.5\%$ EL, $n = 46$ and $n = 42$, respectively, P = 0.35), suggesting that although these genes regulate pulling forces, the cortex in *par-2* and *par-3* mutant embryos cannot simply be regarded as posteriorized or anteriorized with respect to pulling forces before the time of anaphase (see Discussion). It should be noted that *par-2* embryos have a partial rotation defect during centration; we irradiated centrosomes only in embryos that had undergone at minimum of 45° rotation before having reached half the distance to the center. Because it is possible that these embryos represent a special subset of *par-2* embryos in which PAR-2 has partial function, the results of ablations at this stage might not reveal forces in the complete absence of PAR-2. Even with this caveat, based on stages that follow this, we can conclude that the cortical proteins that regulate spindle positioning also function to regulate the forces that we observed.

Discussion

As a step to better understand the regulation of forces acting on the mitotic spindle of *C. elegans* zygotes, we have devised an efficient approach to study how pulling forces are modulated through the cell cycle. We determined that posterior spindle displacement initiates during metaphase, before anaphase onset, and does not depend on anaphase entry. Furthermore, we were able to generate a map of pulling forces throughout the cell cycle and observed that a difference in pulling forces arises early in mitosis and is already present at late prophase and prometaphase. The absence of posterior spindle displacement during this stage is due to a tethering of the spindle by anterior astral microtubules. Together, these results indicate that posterior spindle displacement occurs through a coordinated regulation of different types of forces, pulling and tethering (Fig. 3 D). The regulation of both types of forces is achieved by the cortical PAR proteins. Many asymmetrically localized molecules downstream of PAR proteins have been proposed to regulate pulling forces in the early embryo. GPR-1/2 are redundant activators of G protein signaling required for force generation in anaphase and were shown to be enriched in the posterior half of the embryo (Colombo et al., 2003; Gotta et al., 2003). Likewise, the DEP domain-containing protein LET-99 is enriched in a posterior band and has been proposed to function in opposition to G protein signaling (Tsou et al., 2003). It will be of interest to determine if LET-99, GPR-1/2, and Gα activity impinges on the two types of forces that we observed.

A

B

C
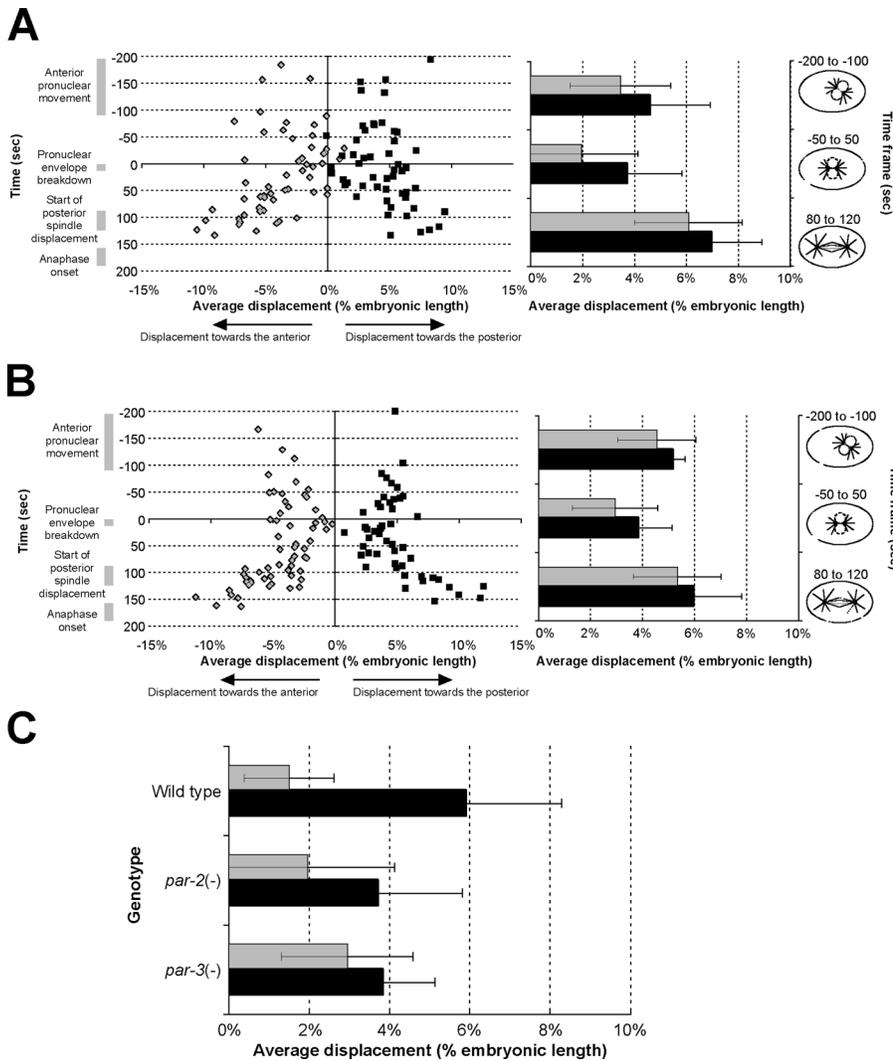
Figure 6. **PAR proteins regulate pulling forces throughout the cell cycle.** Map (left) and quantification (right) of centrosome displacement after OICD in (A) *par-2(lw32)* and (B) *par-3(it71)* mutant embryos. Displacement was determined for anterior centrosomes after posterior centrosome irradiation (gray diamonds and gray bars) and for posterior centrosomes after anterior centrosome irradiation (black squares and black bars). (C) Quantification of anterior (gray bars) and posterior (black bars) centrosome displacement at the time of late prophase/prometaphase in wild-type, *par-2(lw32),* and *par-3(it71)* embryos. Error bars represent SD over, from top to bottom, 19, 16, 21, 25, 21, and 21 embryos, respectively, for each case. The overall displacement in *par-2* mutant embryos ($3.0 \pm 2.1\%$ EL, $n = 46$) was different from wild-type anterior ($1.5 \pm 1.1\%$ EL, $n = 19$, $P = 0.007$) as well as from wild-type posterior ($5.9 \pm 2.4\%$ EL, $n = 16$, $P = 1.3E-5$) centrosomes, and the same was observed when comparing displacement in *par-3* mutant embryos ($3.4 \pm 1.5\%$ EL, $n = 42$) with that of wild-type anterior ($1.5 \pm 1.1\%$ EL, $n = 19$, $P = 1.4E-5$) and wild-type posterior ($5.9 \pm 2.4\%$ EL, $n = 16$, $P = 5.3E-6$) centrosomes.

An interesting observation that we made is that there is a transition in pulling forces in the absence of the metaphase–anaphase transition, suggesting that posterior spindle displacement is regulated in a way that is independent of proteasome activity during this cell cycle transition. This transition might be gradual, or it might be sharp. How does the transition in pulling forces occur? One possibility is that the strength in pulling forces increases at the posterior, thereby overcoming the tethering force at the anterior of the embryo. This could occur through a progressive increase in the number of posterior force generators as the cell cycle progresses. A second possibility is that a change in microtubule dynamics at the anterior of the embryo enables the posterior pulling force to move the spindle posteriorly. Anterior microtubules have been shown to have a higher stability at the anterior cortex compared with the posterior cortex during prometaphase and metaphase (Labbé et al., 2003). These stable microtubules at the anterior of the embryo could contribute to the tethering force that we observed, and a progressive change in microtubule dynamics during progression through the cell cycle could occur to alter the balance of forces on each side of the spindle. It should be noted that the two possibilities we propose are not mutually exclusive. It will
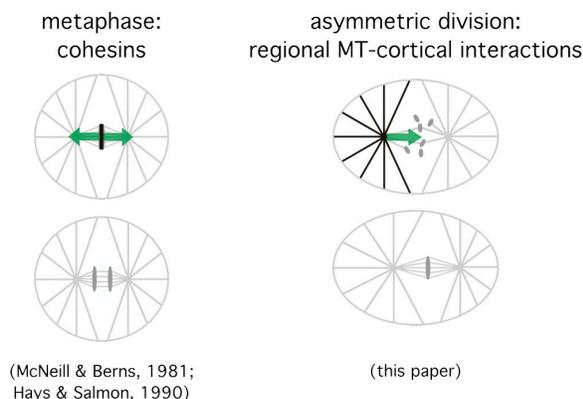
be of interest to determine the relationship between microtubule dynamics and the tethering and pulling forces during progression through the cell cycle.

We have observed that spindle microtubules do not undergo significant poleward flux during mitotic anaphase. This result, together with the observation that chromosome segregation occurs without anaphase A (Oegema et al., 2001), suggests that spindle microtubule dynamics are regulated differently in *C. elegans* zygotes compared with mammalian and *Drosophila* cells, which have been shown to undergo poleward flux (Desai et al., 1998; Brust-Mascher and Scholey, 2002; Maddox et al., 2002). Because the spindle midzone was shown to limit the rate of anaphase pole separation (Grill et al., 2001), this further suggests that the forces responsible for mediating pulling on each aster, and for positioning the spindle, are also involved in segregating chromosomes in the *C. elegans* zygote. Poleward flux and microtubule plus end dynamics have been proposed to be responsible for generating tension at the kinetochores (Mitchison and Salmon, 1992; Waters et al., 1996; Maddox et al., 2003), and an asymmetry in astral microtubule flux during late prophase and prometaphase could potentially account for the early asymmetry in pulling forces that we observed in early *C.*

elegans embryos. However, we are currently unable to image individual astral microtubules long enough in vivo, by conventional confocal microscopy, to determine whether or not they undergo flux. It also remains to be established whether or not the absence of significant poleward flux in spindle microtubules that we measured is specific to *C. elegans* zygotes or is a more general property of asymmetrically dividing cells. In this sense, it is interesting to note that spindle microtubules do not undergo poleward flux in *S. cerevisiae*, which also divides asymmetrically (Maddox et al., 2000).

Chromosome segregation can occur when no pulling force is measured, such as in embryos in which the functions of genes encoding the two Gα subunits, GOA-1 and GPA-16, are disrupted (Gotta and Ahringer, 2001; Grill et al., 2003), or when astral microtubules are largely defective, such as in embryos lacking the function of the doublecortin-like kinase protein ZYG-8 (Gönczy et al., 2001). This finding suggests that there could be a redundant mechanism that enables the spindle to undergo anaphase even when tension is low or absent at the poles. For example, antiparallel sliding by overlapping polar microtubules, which likely limits anaphase pole separation in the presence of pulling forces from astral microtubules (Grill et al., 2001), might still occur in the absence of these pulling forces and would be expected to result in centrosome separation without necessarily producing displacement of the spindle to the posterior. Such a redundant mechanism might function to ensure that chromosome segregation occurs at a constant rate, even when minor perturbations in the strength of the astral pulling forces are present.

Transient tethers

metaphase:
cohesins

asymmetric division:
regional MT-cortical interactions

(McNeill & Berns, 1981;
Hays & Salmon, 1990)

(this paper)

Figure 7. **Model depicting the transient tethers that act during mitosis in cells that divide symmetrically and early *C. elegans* embryos, which divide asymmetrically.** In this model, the net pulling vectorial forces are depicted as arrows and tethers are depicted in dark. In cells that divide symmetrically, tension is present at the kinetochores during metaphase and forces are kept at equilibrium by cohesins that link sister chromatids (McNeill and Berns, 1981; Hays and Salmon, 1990). Cohesins are degraded at anaphase onset, allowing chromosome segregation to occur. In asymmetrically dividing *C. elegans* embryos, a posterior pulling force is present during late prophase and prometaphase, and this force is kept at equilibrium by the tethering of astral microtubules at the anterior of the embryo. This tether is released during metaphase, allowing posterior movement of the spindle. MT, microtubule.

Finally, our results provide an interesting parallel between cells that divide symmetrically and those that divide asymmetrically (Fig. 7). In symmetrically dividing cells, poleward microtubule flux and microtubule plus end dynamics generate forces at the kinetochores before anaphase onset (Mitchison and Salmon, 1992; Waters et al., 1996), and cohesion between sister chromatids contributes to a tethering force that temporarily prevents chromosome segregation. Likewise, in *C. elegans* embryos, we observed an asymmetry in the spindle-positioning pulling forces before anaphase, and anterior astral microtubules contribute to a tethering force that temporarily prevents posterior movement of the spindle. Although chromosome segregation and anaphase entry are regulated by components of the spindle checkpoint, it is not clear whether such a checkpoint exists to regulate the timing of posterior spindle displacement. Such a spindle-positioning checkpoint could be required to ensure that posterior spindle displacement does not initiate before all kinetochores have made microtubule attachments, thus ensuring fidelity of chromosome segregation. The difference between symmetric and asymmetric cell divisions lies in a polarization of the cell before entry into mitosis. Therefore, it is possible that the components required to establish and maintain cell polarity, which are active in asymmetrically dividing cells, are impeding on the cell cycle machinery in ways that have yet to be described. In support of this possibility, it should be noted that disrupting the function of the polarity genes *par-2* and *par-3* in *C. elegans* affects the progression of embryos through mitosis, prolonging the time between anaphase onset and cytokinetic furrow ingression (Kirby et al., 1990; unpublished data). It will be of interest to study in more detail links between polarity and cell cycle in the future.

Materials and methods

Laser irradiations

For centrosome irradiations, embryos were obtained by cutting open gravid hermaphrodites using two 25-gauge needles. Embryos were handled individually and mounted on a coverslip coated with 1% poly-L-lysine in 1 µl of egg buffer. The coverslip was placed on a 3% agarose pad and the edge was sealed with petroleum jelly. This mounting method has been shown previously to be compatible with normal development, and thus does not disrupt cellular processes (Sulston et al., 1983). The visualization of embryos by DIC microscopy was done using a video camera (model C2400-07 Newvicon; Hamamatsu Photonics) mounted on a microscope (model Eclipse 800; Nikon). At a given time, the anterior or posterior centrosome was irradiated with several laser pulses using a 2-mW pulsed nitrogen laser (model VSL-337; Laser Science Inc.), exciting Coumarin 440 dye in a lasing chamber (Photonic Instruments), to deliver 436-nm laser pulses to cells for 10 to 20 s. Centrosome disruption was assessed by monitoring changes in the morphology of the targeted centrosome. Time-lapse images were acquired at 2-s intervals using 4D Grabber (Integrated Microscopy Resource, University of Wisconsin, Madison, WI), analyzed with NIH Image (National Institutes of Health) and Microsoft Excel, and processed with Adobe Photoshop.

Estimations of relative pulling forces

Relative forces applied on the asters were estimated by measuring the distance traveled by centrosomes following irradiation. Centrosome positions were averaged for five time frames from 20 to 10 s before irradiation and five time frames from 20 to 30 s after the irradiation. The difference in average position before and after centrosome irradiation was divided by the length of the embryo and expressed as a percentage of EL. Because irradiation was performed over a broad time scale, we used the irradiation midpoint as the effective time of irradiation. Calculating peak velocity of cen-

trosomes after irradiation gave similar results as measuring distance traveled to estimate relative force on each centrosome.

Online supplemental material

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