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PAR-dependent and geometry-dependent mechanisms of spindle positioning

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During intrinsically asymmetric division, the spindle is oriented onto a polarized axis specified by a group of conserved PAR proteins. Extrinsic geometric asymmetry generated by cell shape also affects spindle orientation in some systems, but how intrinsic and extrinsic mechanisms coexist without interfering with each other is unknown. In some asymmetrically dividing cells of the wild-type *Caenorhabditis elegans* embryo, nuclear rotation directed toward the anterior cortex orients the forming spindle. We find that in such cells, a PAR-dependent mechanism dominates and causes rotation onto the polarized axis, regardless of cell shape. However, when geometric asymmetry is removed, free nuclear rotation in the center of the cell is observed, indicating that the anterior-directed nature of rotation in unaltered embryos is an effect of cell shape. This free rotation is inconsistent with the prevailing model for nuclear rotation, the specialized cortical site model. In contrast, in *par-3* mutant embryos, a geometry-dependent mechanism becomes active and causes directed nuclear rotation. These results lead to the model that in wild-type embryos both PAR-3 and PAR-2 are essential for nuclear rotation in asymmetrically dividing cells, but that PAR-3 inhibits geometry-dependent rotation in nonpolarized cells, thus preventing cell shape from interfering with spindle orientation.

Introduction

Proper orientation of the mitotic spindle is important for development and growth in a number of organisms. In budding yeast, the spindle must be oriented into the bud neck to ensure the proper segregation of daughter nuclei (Schuyler and Pellman, 2001; Segal and Bloom, 2001). In animal cells, the position of the spindle determines the division plane, which is critical for several processes including maintaining tissue organization, positioning cells for cell signaling, and dividing asymmetrically to produce daughters with different cell fates (Rhyu and Knoblich, 1995; Doe and Bowerman, 2001). Such asymmetric cell division depends on coordinating the position of the mitotic spindle with the axis of cellular polarity. A number of studies have revealed genetic pathways that regulate spindle orientation within cells (for review see Rose and Kemphues, 1998b; Doe and Bowerman, 2001). In addition to these intrinsic mechanisms, cell division in both asymmetrically and symmetrically dividing cells can be influenced by cell shape induced by cell contact or egg shell constraints (Freeman, 1983; Symes and Weisblat, 1992; Wang et al., 1997; O'Connell and Wang, 2000; Tsou et al., 2002). The physical and molecular mechanisms by which cell geometry affects spindle alignment and the biological relevance of such effects have rarely been addressed (O'Connell and Wang, 2000). Cell shape effects on spindle orientation could positively or negatively contribute to the developmental program. In particular, the effects of cell geometry and intrinsic polarity could cooperate with each other to specify a certain spindle position, or alternatively, they could compete and interfere with each other. In the case of negative competition, mechanisms by which cells are able to resist or override geometric effects would appear essential to maintain the division pattern for normal development. However, mechanisms by which cells avoid geometric effects or integrate them with asymmetric division have not been described.

The *Caenorhabditis elegans* embryo is an excellent system in which to study spindle orientation, as it displays both symmetric and asymmetric divisions in a virtually invariant pattern. The spindle in the one-cell embryo (P_0) orients onto the longitudinal axis of the embryo, which is also the polarized anterior/posterior axis. Division is asymmetric, producing an anterior AB cell and a posterior P_1 cell. At second cleavage, AB divides symmetrically with a transverse spindle, while the P_1 spindle is oriented again on the anterior/

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posterior axis (Rose and Kemphues, 1998b; Bowerman and Shelton, 1999). Longitudinal spindle orientation in P_0 and P_1 results from a 90° rotation of the nuclear–centrosome complex during prophase, which does not occur in AB.

Nuclear rotation in asymmetrically dividing cells is under the control of polarity cues. Polarity in the *C. elegans* embryos is established in the one-cell embryo through the asymmetric distributions of several PAR proteins, which are conserved in many organisms (Ohno, 2001). PAR-3 and PAR-2 are present at the anterior and posterior cortex, respectively, of both P_0 and P_1; PAR-3 is also present uniformly at the cortex of AB (Etemad-Moghadam et al., 1995; Boyd et al., 1996). Previous studies showed that nuclear rotation occurs in *par-3* and *par-3;par-2 double* mutants in both one- and two-cell embryos, but not in *par-2* single mutants (Cheng et al., 1995). These results combined with immunolocalization studies led to the model that in wild-type embryos, neither PAR-3 nor PAR-2 is required for rotation. Rather, it was proposed that PAR-3 somehow inhibits rotation in AB and the role of PAR-2 is to restrict the localization of PAR-3 to the AB cell cortex and the anterior cortex of P_1 (Cheng et al., 1995; Etemad-Moghadam et al., 1995). However, this model cannot explain how nuclear rotation occurs in *par-3* embryos where there is no apparent cellular polarity.

The molecular mechanism of nuclear rotation and how it is regulated by PAR proteins remains to be elucidated. In both the P_0 and P_1 cells, a process called centration occurs where nuclei migrate from the posterior to the center of the cell. During this time, the nuclear–centrosome complex usually begins rotation, and rotation is completed before nuclear envelope breakdown. Nuclear rotation depends on the function of the microtubule motor dynein in P_0 cells. In P_1 cells, the dynein-associated dynactin complex accumulates at a site on the anterior cortex, coincident with the position of the midbody/cell division remnant at the cell contact between P_1 and AB. It has been proposed that dynein present at this dynactin-enriched cortical site captures and shortens astral microtubules, generating a pulling force that causes rotation and an extended anterior movement of the nucleus away from the center of cells (Fig. 1 A; Hyman and White, 1987; Hyman, 1989; Waddle et al., 1994; Keating and White, 1998; Skop and White, 1998; Gönczy et al., 1999; Gönczy, 2002). We refer to this type of movement as directed rotation, because the rotation appears directed toward the cell contact region and the nucleus becomes closely juxtaposed to the membrane. The observation that directed rotation occurs in both cells of *par-3* embryos has been interpreted as evidence for cortical site activity in both cells (Waddle et al., 1994; Keating and White, 1998). However, in wild-type embryos, PAR-3 is present on both the AB and P_1 side of the cell contact region by the time of rotation, and thus it is not clear how PAR-3 could inhibit the accumulation of dynactin and/or its function only in AB. Thus, although the cortical site model explains P_1 rotation, how this cortical site is regulated by PAR polarity is unknown. Also, no such specialized site has been identified in P_0, and there is no movement of the nucleus past the center of the cell during rotation in P_0.

Recently, a second model for rotation was proposed based on the unique localization pattern of the LET-99 protein (Fig. 1 B), which functions as an intermediate between PAR polarity cues and spindle positioning (Tsou et al., 2002). LET-99 is required for rotation, and is enriched in a cortical band in P cells in a PAR-2/PAR-3–dependent manner. It was proposed that the effect of LET-99 activity is a reduction of force generation at the cortex in the region of the band. Thus, the forces on astral microtubules outside the LET-99 band will be stronger, generating a free central rotational movement of the nucleus (Fig. 1 B) that is different from the anteriorly directed rotation predicted by the cortical site model. The LET-99 band model provides a common mechanism for generating nuclear rotation in both P_0 and

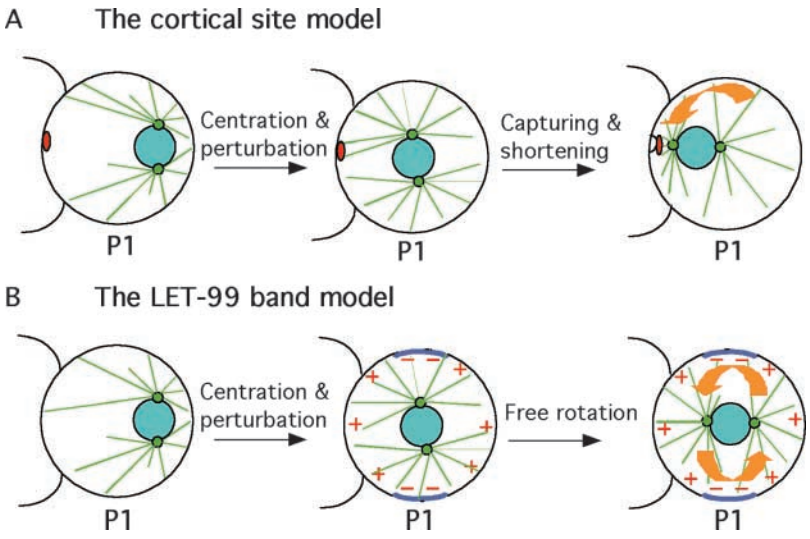


Figure 1. **Two models for nuclear rotation.** P_1 blastomeres are shown; the region of cell contact with AB, which is anterior, is to the left. Microtubules and centrosomes are shown in green. (A) The cortical site model. The anterior cortical site enriched for dynactin is shown in red. During movement of the nucleus to the center of the cell (centration) any slight perturbation that tilts the nuclear–centrosome complex results in capture of microtubules from one centrosome; capture and shortening of microtubules causes nuclear rotation directed toward the cortical site and results in one centrosome being closely associated with the anterior cortex. (B) The LET-99 band model. Thick blue lines indicate the peripheral region enriched for LET-99 that appears during prophase as a band encircling the P cells. The proposed effect of the LET-99 band is to decrease force between the cortex and astral microtubules (−), compared with the force at the remainder of the periphery (+). After centration, any stochastic tilt of the nuclear–centrosome complex would lead to free rotational movement with the nucleus located centrally and the two centrosomes equidistant from anterior and posterior.

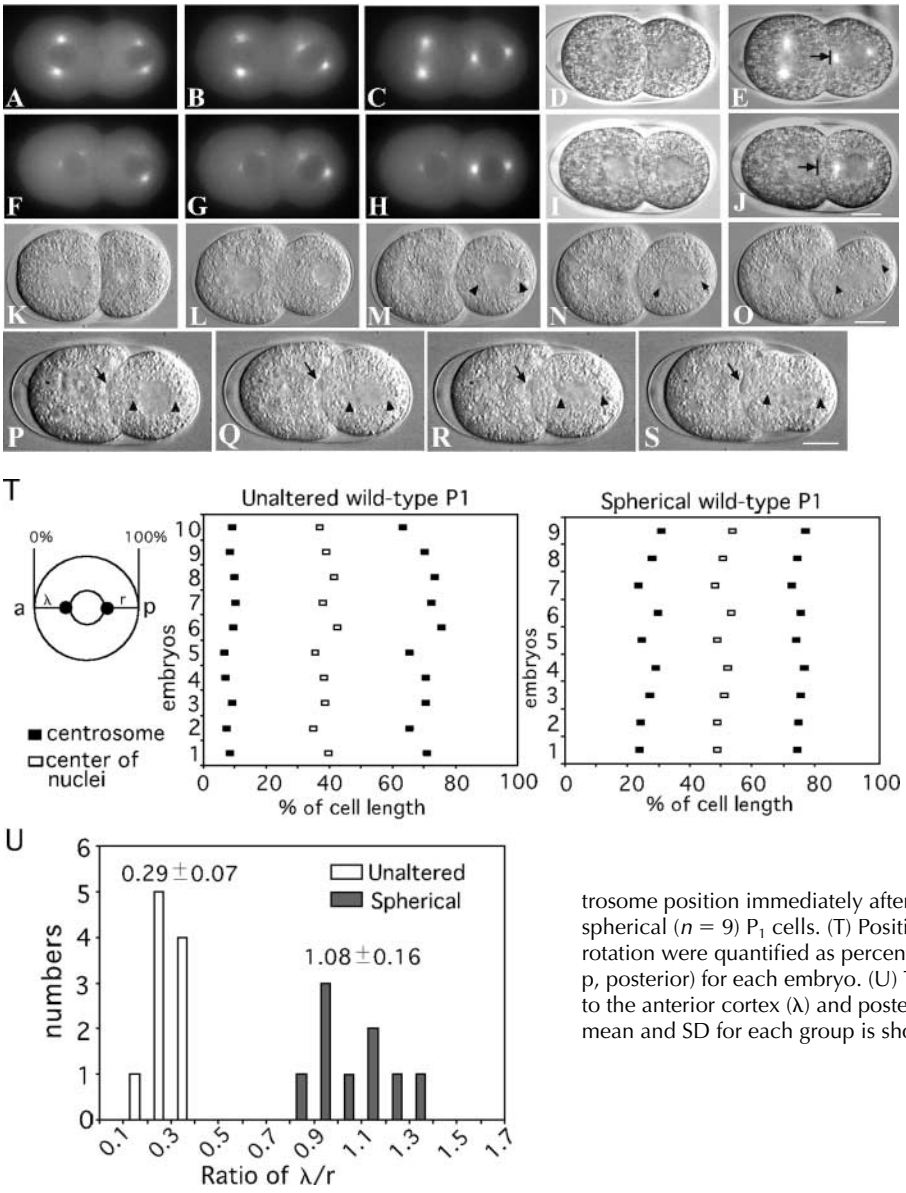


Figure 2. **Wild-type spherical P_1 cells show free nuclear rotation unaffected by geometry.** Time-lapse video microscopy series of unaltered (A–E) and spherical (F–S) wild-type P_1 blastomeres; embryo in series K–O has an ectopic flat surface on top. Each row shows a single embryo. Anterior is to the left in this and all subsequent figures. Centrosomes were visualized with GFP-labeled tubulin (A–C and F–H), or by DIC microscopy. (A–J) Comparison of nuclear rotation and centrosome positioning in unaltered (A–E) and spherical (F–J) P_1 cells. DIC images (D and I) were taken immediately after the last epifluorescence image, and merged (E and J) to show the relative position of the centrosomes to the cortex. Arrows and bars (E and J) indicate the anterior cortex of P_1 cells. (K–O) Wild-type embryo mounted on agar; the posterior blastomere is "spherical" in terms of the cell contact region, but had an ectopic flat side on top (parallel with the page) from the weight of the coverslip. Arrowheads (M and N) indicate centrosome position after nuclear rotation. (P–S) Higher magnification of a wild-type embryo with a spherical P_1 after nuclear rotation, showing a membrane invagination (arrow) that protrudes into the AB cell but not the P_1. Arrowheads indicate centrosome position. (T and U) Quantitative analysis of the nuclear and centrosome position immediately after nuclear rotation in unaltered ($n = 10$) and spherical ($n = 9$) P_1 cells. (T) Positions of centrosomes and nuclei after nuclear rotation were quantified as percentage of cell length (see diagram; a, anterior; p, posterior) for each embryo. (U) The ratios of the distances from centrosomes to the anterior cortex (λ) and posterior cortex (r) were expressed as λ/r. The mean and SD for each group is shown. Bars, 10 μm.

P_1. However, although the link between the LET-99 band and PAR polarity is clear, this model cannot explain how nuclear rotation occurs in *par-3* cells where the LET-99 band is not present.

Thus, a clear framework for how the PAR proteins regulate nuclear rotation has not been produced based on the current data. This suggests that other factors that influence spindle orientation remain to be found. Previously, we showed that nuclear rotation in one-cell *par-3* embryos is not equivalent to wild-type rotation, but rather is due to an extrinsic cue, the oblong shape of the embryo (Tsou et al., 2002). In this paper, we re-evaluate the mechanism of nuclear rotation in both wild-type and *par-3* two-cell embryos. The results challenge the cortical site model and support the LET-99 band model. Furthermore, the data clarify the role of the PAR proteins and indicates that PAR-3 has two roles: (1) it is required for an intrinsically programmed mechanism of nuclear rotation in asymmetrically dividing cells; and (2) is also required to inhibit geometry-dependent rotation in nonpolarized cells.

Results

Cell shape is not required for and does not affect centration, nuclear rotation, and asymmetric spindle elongation in wild-type P_1 cells

In wild-type *C. elegans* embryos, the cells are not spherical at the early two-cell stage, but rather have a flat surface at the region of cell contact. As the cell cycle proceeds and the AB cell starts dividing before the P_1 cell, the cell contact region curves slightly into the P_1 cell. The flat/curved-in shape of the cell contact region is a geometric asymmetry that could influence spindle positioning. To test the role of this asymmetry in nuclear rotation in wild-type embryos, we manipulated the cell shape of P_1 cells. Although removal of the eggshell and vitelline envelope results in spherical P_0 cells, the blastomeres in multicellular embryos still have flat surfaces at cell contact regions (unpublished data). Therefore, we devised a new method to generate spherical blastomeres. In early two-cell embryos, the AB blastomere was irradiated with UV light. In such embryos, the AB cell cycle was re-

tarded, but the untreated P_1 cell divided as normal. As the cell cycle proceeded, the P_1 cell rounded up and the cell contact region curved into the irradiated AB blastomere, eliminating the flatness of the cell contact region (Fig. 2). This is almost opposite that of the unaltered two-cell embryo, where AB is slightly spherical and P_1 is slightly curved in at the time of nuclear rotation (compare Fig. 2 E with Fig. 2 J). For simplicity, we refer to the altered shape of a P_1 cell under these conditions as spherical, although the cell may not be a perfect sphere.

In wild-type spherical P_1 cells, nuclear centration and rotation occurred (Table I; Fig. 2, F–J), followed by normal asymmetric elongation of the anaphase spindle (Fig. 2, R and S). This result indicates that UV treatment does not cause damage that affects spindle positioning, and further shows that the cell shape observed in normal wild-type P_1 cells is not required for nuclear rotation and asymmetric spindle elongation. To further test whether cell shape affects nuclear rotation in wild-type backgrounds, additional geometric asymmetry was ectopically created by placing embryos on agar pads under coverslips, which results in a flat surface on top of the embryo (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb200209079/DC1). The flatness of the cell contact region was then eliminated by irradiating the adjacent blastomere as described above. In such wild-type P_1 cells, nuclear rotation still occurred and spindles elongated asymmetrically (Fig. 2, K–O; Table I). Thus, nuclear rotation in wild-type spherical P_1 cells is resistant to ectopic changes in cell shape.

Membrane invagination and extended anterior movement of the nucleus are cell shape-dependent and are not required for centration, nuclear rotation, and asymmetric spindle elongation in wild-type P_1 cells

During nuclear rotation in wild-type P_1 cells, the appearance of a membrane invagination at the anterior cortex adjacent to the cell division remnant is often seen at the time of nuclear rotation (Hyman, 1989). During rotation, the anterior centrosome moves further toward the invagination, resulting in the close juxtaposition of the nucleus to the anterior cortex (Fig. 1 A; Fig. 2, A–E). This membrane invagination is thought to be indicative of tension on the cortex and has been considered as evidence for a localized cortical site that pulls the nuclear–centrosome complex toward the anterior during rotation (Fig. 1 A; Hyman, 1989; Keating and White, 1998). If such a localized cortical site drives nuclear rotation in wild-type P_1 cells by capture and shortening of microtubules as proposed, then the close juxtaposition of the nucleus to the anterior cortex after rotation, as well as the membrane invagination, should be independent of cell shape. To test this, we followed the position of nuclei during nuclear rotation in spherical wild-type P_1 cells. Surprisingly, although centration and nuclear rotation occurred normally as described above, the juxtaposition of the nucleus to the anterior cortex was never observed in spherical wild-type P_1 cells (Fig. 2, F–J, P, and Q). Instead, quantification showed that immediately after rotation, the nucleus was always located at the center of the cell (Fig. 2, T and U). The nucleus remained in this position until anaphase, when the spindle

Table I. **Effects of cell shape on spindle orientation in two-cell embryos**

	Unaltered P_1 (1-side flat)			Spherical P_1			Spherical P_1/flattened on top		
Wild type	100%	0%	0%	100%	0%	0%	100%	0%	0%
	n = 13			*n* = 12			*n* = 13		
par-3	100%	0%	0%	35%	27%	38%	0%	0%	100%
	n = 47			*n* = 26			*n* = 24		

Spindle orientation was scored at nuclear envelope breakdown (the time at which all wild-type unaltered P_1 cells have undergone nuclear rotation) under three different conditions as described in the text. The angle of orientation was determined as the angle between the longitudinal (anterior/posterior) axis of the embryo (0°) and a line passing through both centrosomes. For each genotype and condition shown, the percentage of embryos scored as having longitudinal (horizontal line in P_1 cell of embryo diagram), dorsal/ventral (vertical line), and left/right (aster, i.e., oriented into the plane of the paper) spindle orientations is shown. All nuclear–centrosome complexes oriented within 45° of the longitudinal axis were grouped as longitudinal. All those between 45 and 90° were grouped as either dorsal/ventral or left/right, which together are referred to as transverse in the text. The exact spindle positions for individual embryos are shown below the percentages. For longitudinal and dorsal/ventral orientations, the angle of orientation is shown as a line on a quadrant (longitudinal, 0°; dorsal/ventral, 90°). For left/right orientations, dots in a circle indicate the position of the spindle aster that was oriented towards the coverslip. The total number of embryos (*n*) scored for each condition is shown. For *par-3* embryos, both the AB and P_1 behaved the same under all conditions, and the results were pooled. Longitudinal orientations in spherical *par-3* embryos were due to centrosome migration onto the longitudinal axis, as described in the text for unaltered *par-3* embryos, and transverse orientations close to 45° were due to less severe centrosome mispositioning. In no cases was the angle due to a rotational movement of the nucleus.

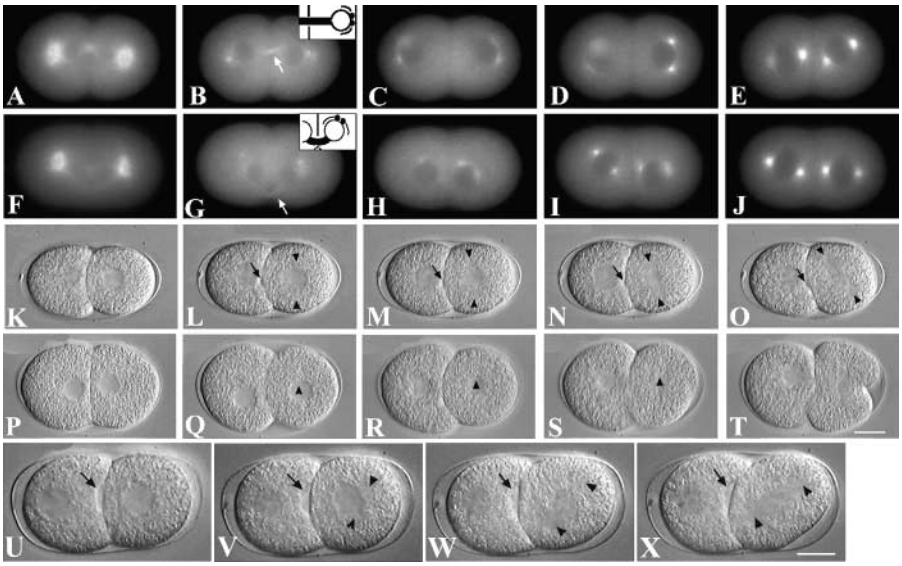


Figure 3. **Nuclear rotation in *par-3* embryos is a consequence of geometric effects.** Time-lapse video microscopy series of unaltered (A–J) and spherical (F–X) *par-3* blastomeres; embryo in series P–T has an ectopic flat surface on top. Each row shows a single embryo. Centrosomes were visualized with GFP-labeled tubulin (A–J), or by DIC microscopy. (A–E) Embryo in which the centrosomes in each blastomere migrated onto a transverse axis (C and D), and then nuclear rotation directed toward the cell contact region occurred (E). (F–J) Embryo in which the central spindle was displaced laterally (compare arrows in G and B) at first cleavage, resulting in mispositioned centrosomes (compare insets). The centrosomes migrated onto the longitudinal axis (H and I), giving rise to a longitudinal spindle in the absence of rotation (J). (K–O) Embryo with a spherical posterior blastomere. Centrosomes (arrowheads) migrated onto a transverse axis (N) and no rotation occurred, resulting in a transverse spindle (O). (P–T) Embryo mounted on agar; the posterior blastomere is "spherical" in terms of the cell contact region, but had an ectopic flat side on top from the weight of the coverslip. The nuclear–centrosome complex oriented toward the coverslip (R, one centrosome is visible; arrowhead); the nucleus was also closer to the coverslip. As spindle elongation occurred, the spindle moved onto the other transverse axis, which is the long axis of the cell under these flattened conditions. (U–X) Higher magnification of an embryo with a spherical posterior blastomere. A membrane invagination (arrow) protrudes into the left blastomere in this embryo, as well as in the embryo shown in K–O. Arrowheads indicate centrosome position. No nuclear rotation during prophase was observed, but the spindle skewed onto an oblique axis at it elongated. Bars, 10 μm.

asymmetrically elongated toward the posterior as in unaltered P_1 cells. These results indicate that the extended anterior movement of the nucleus during rotation is not essential for centration, nuclear rotation, or asymmetric elongation of spindles. Rotation in the absence of extended anterior movement (which we refer to as free nuclear rotation) is inconsistent with the prediction of the cortical site model. Instead, these results suggest the final position of the nucleus in wild-type P_1 cells is cell shape-dependent.

Interestingly, although membrane invaginations were never observed in spherical P_1 cells ($n = 12$), membrane invaginations were frequently observed in the irradiated AB cells ($n = 7/12$). As stated earlier, the irradiation severely slowed down the AB cell cycle and prevented normal spindle formation. Membrane invaginations in AB cells appeared as the cell contact region curved into the AB cell concomitant with mitosis in the spherical P_1 cell. The invaginations were centered around the cell division remnant (identified from the position of the midbody at first cytokinesis; Fig. 2, P–S). This observation shows that membrane invaginations are able to form in these embryos, indicating that UV irradiation did not damage this process. Examination of the P_1 cell in embryos with AB membrane invaginations showed that the centrosomes and spindles of P_1 cells did not move or point toward the invagination during nuclear rotation and spindle elongation (Fig. 2, P–S). These results indicate that the formation of a membrane invagination is independent of nuclear rotation and asymmetric spindle elongation in spherical P_1 cells. Furthermore, invaginations in untreated wild-type embryos appear in P_1 (Hyman, 1989), which has a flat or curved-in cell contact region, and invaginations in irradiated embryos only appeared in the irradiated AB cells with curved-in cell contact regions. These observations suggest that the invaginations are cell shape-dependent.

These results indicate that both the anterior position of the nucleus and the corresponding membrane invagination during rotation in wild-type P_1 cells can be explained as a consequence of cell geometry, rather than being causally linked to the mechanism of rotation. Further, all of the observations on spherical wild-type P_1 cells described are inconsistent with the cortical site model because the free nuclear rotation observed in spherical P_1 cells cannot be explained by this model, regardless of whether the cortical site is present or not. Rather, these results indicate that nuclear rotation in P_1 cells is controlled by mechanisms that produce free nuclear rotation, similar to the rotation observed in P_0 cells.

Nuclear rotation does not occur in spherical *par-3* cells at the two-cell stage

It has been proposed that the specialized cortical site drives nuclear rotation in both blastomeres of two-cell *par-3* embryos. However, the results above indicate that the cortical site is not required for nuclear rotation in wild-type cells where rotation is under the control of polarity cues. This raises the question of what causes nuclear rotation in *par-3* two-cell embryos in the absence of polarity. We have previously shown that nuclear rotation in one-cell *par-3* embryos is driven by cell shape (Tsou et al., 2002). To determine whether geometric asymmetry also causes nuclear rotation in *par-3* two-cell embryos, we compared centrosome movements in *par-3* embryos with normal and altered cell shapes.

To more carefully analyze nuclear rotation in unaltered *par-3* embryos, we first traced centrosome movements us-

ing live imaging of GFP-tubulin, and RNA interference (RNAi)* to produce the *par-3* phenotype in two-cell embryos. As previously reported for *par-3(it71)* mutants (Cheng et al., 1995), in *par-3(RNAi)* two-cell embryos, spindles in both cells aligned toward the cell contact region in all embryos ($n = 47$); this orientation will be referred to as longitudinal (Table I). In 64% of these embryos, longitudinal spindle orientation resulted from an apparently wild-type nuclear rotation where one of the centrosomes moved directly toward the cell contact region (Fig. 3, A–E). However, in 36% of the cases (17/47), no nuclear rotation was observed. Rather, the centrosomes were already aligned along the long axis of the embryo as soon as they became visible (Fig. 3, F–J). In such embryos, displacement of the first cleavage spindle resulted in mispositioned centrosomes whose daughters migrated directly onto the longitudinal axis (Fig. 3, G–I). Thus, longitudinal spindle orientation in a subset of *par-3* embryos is due to abnormal centrosome positioning instead of directed nuclear rotation.

To test the role of cell shape in nuclear rotation in *par-3* mutant embryos, we next generated spherical blastomeres at the two-cell stage using the method described above for wild type. Strikingly, in spherical *par-3(it71)* P_1 or AB cells, nuclear rotation was never observed ($n = 26$; Table I and Fig. 3, K–N). In 65% of these cells, spindles initially set up transversely on either the dorsal/ventral or left/right axes (Fig. 3 O; Table I), although as anaphase proceeded, spindles often skewed onto a more oblique axis. Although spindles set up longitudinally in the remaining 35% of embryos, this was not due to nuclear rotation, but rather to abnormal centrosome positioning as observed in a similar proportion of unaltered *par-3* embryos (Table I). In all the spherical *par-3* two-cell blastomeres, regardless of where spindles were oriented, the spindle poles rocked vigorously during anaphase (unpublished data) as observed in untreated *par-3* cells (Cheng et al., 1995). This observation suggests that as in wild-type spherical P_1 cells where rotation did occur, interactions between astral microtubules and the cortex in *par-3* cells were not damaged by UV exposure. In addition to the failure of rotation, neither membrane invaginations nor extended anterior cortex–directed movements of the nucleus were observed in spherical *par-3* cells ($n = 26$). Interestingly however, just as in irradiated wild-type embryos, membrane invaginations were frequently seen ($n = 15/26$) in the irradiated blastomeres with the curved-in cell contact region (Fig. 3, U–X). These results show once again that the UV irradiation did not damage the process of membrane invagination (an indicator of the cortical site) and support the view that membrane invagination is cell shape-dependent. Together, these results suggest that nuclear rotation in unaltered nonspherical *par-3* two-cell embryos is due to extrinsic geometric asymmetry, and not the normal intrinsic mechanism for nuclear rotation. This supports the hypothesis that there is an intrinsic mechanism that causes free nuclear rotation in wild-type embryos that is PAR polarity–dependent.

*Abbreviations used in this paper: DIC, differential interference contrast; RNAi, RNA interference.

Nuclear rotation toward ectopic flat sides occurs in *par-3* embryos

To further test the hypothesis that cell shape can ectopically orient nuclear–centrosome complexes in the absence of polarity in *par-3* cells, additional geometric asymmetry was created by placing embryos on agar pads under coverslips, which results in a flat surface on top of the embryo (Fig. S1). The flatness of the cell contact region was then eliminated by irradiating the adjacent blastomere as described before. In such *par-3* embryos, the nuclear–centrosome complex always oriented toward the flat surface/coverslip during prophase (Fig. 3, P–S; Table I). Therefore, the spindle set up perpendicular to the flat surface, but as the spindle elongated during anaphase, it then moved within the cytoplasm into a plane parallel to the flat coverslip (Fig. 3, S–T). The orientation of the nuclear–centrosome complex toward the flat surface occurred in both *par-3* blastomeres tested (AB or P_1), and even in those *par-3* embryos where the initial centrosome position was longitudinal due to centrosome mispositioning. We conclude that nuclear rotation in two-cell *par-3* embryos is entirely driven by the asymmetry of cell shape, which argues against the model that nuclear rotation in *par-3* embryos is due to a specific cortical site at the cell contact region or any other intrinsic asymmetry. Further, these results clearly show that cell shape, which does not have any effect on nuclear/spindle positioning in polarized wild-type P_1 cells, has a dramatic influence on spindle behaviors in nonpolarized *par-3* embryos. This indicates that a PAR polarity–dependent mechanism exists in wild-type P_1 cells that overrides the effects of geometric asymmetry generated by cell shape, and causes spindles to orient onto the polarized anterior/posterior axis.

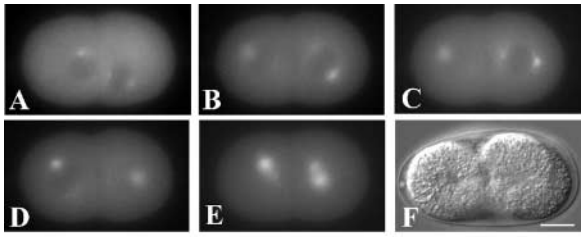
PAR-3 is required for wild-type nonpolarized cells to avoid geometry-driven nuclear rotation

As shown above, geometric asymmetry generated by the flat cell contact region or an ectopic flat surface can induce ectopic nuclear rotation in nonpolarized *par-3* cells. However, in wild-type AB cells, which are also nonpolarized cells but have PAR-3 present all around the cortex, the flat contact region does not induce ectopic nuclear rotation. These correlations lead to a hypothesis that the uniform distribution of PAR-3 around the cortex is required for wild-type nonpolarized cells to avoid the effects of cell shape, and further, that abnormal PAR-3 localization could inhibit geometry-driven rotation in mutant backgrounds. We have shown that loss of PAR-3 in *par-3* mutant embryos causes sensitivity of the nuclear/spindle positioning to the cell shape effect in both one- and two-cell embryos (this paper and Tsou et al., 2002). To further test the hypothesis, we examined the converse situation in which PAR-3 is ectopically expressed. In *par-2* mutant embryos, PAR-3 is localized around the entire cortex in the P_0 and in both cells of the two-cell embryos (Etemad-Moghadam et al., 1995); our hypothesis predicts that cells in *par-2* embryos should be resistant to the effects of geometry. In unaltered *par-2(lw32)* embryos, nuclear rotation failed half of the time in one-cell embryos (5/11) and failed completely in two-cell *par-2* embryos ($n = 26$; Cheng et al., 1995), consistent with this hypothesis. Further, when the ectopically localized PAR-3 was removed in *par-3(it71);par-*

2(RNAi) embryos, nuclear rotation resumed in both one- and two-cell embryos ($n = 11$; Cheng et al., 1995). These results suggest that nuclei/spindles in cells with ectopic expression of PAR-3 are resistant to the effects of geometry. To confirm that the nuclear rotation in *par-3(it71);par-2(RNAi)* embryos is geometry-dependent rotation, we generated spherical cells at the two-cell stage. In such spherical *par-3;par-2* cells, nuclear rotation directed toward the cell contact region was never observed ($n = 14$). The spindles set up transversely in the majority of embryos (10/14). As described earlier for *par-3* mutant embryos, in a subset of *par-3;par-2* embryos (4/14) spindles formed on the longitudinal axis without nuclear rotation. Finally, in spherical two-cell *par-3;par-2* blastomeres with an ectopic flat side created by a coverslip, the nucleus oriented toward the flat side during prophase in all embryos ($n = 14$) as described earlier for *par-3* single mutants. Together, these results support the hypothesis that PAR-3 is required for wild-type AB lineage cells to avoid the negative influence of cell shape that can ectopically orient spindles. Further, these results indicate that ectopic PAR-3 (as seen in *par-2* embryos) is inhibiting geometry-driven nuclear rotation rather than the intrinsic mechanism that drives wild-type P_1 nuclear rotation. Therefore, we conclude that in contrast to previous interpretations (Cheng et al., 1995), both PAR-2 and PAR-3 are essential for the intrinsically programmed nuclear rotation event that occurs in wild-type polarized P lineage cells. However, neither PAR-3 nor PAR-2 is required for geometry-dependent nuclear rotation, and indeed, PAR-3 somehow inhibits such geometry-dependent rotation.

LET-99 is required for geometry-driven nuclear rotation in *par-3* mutant embryos

We showed above that PAR-3 is required for nonpolarized AB cells to be resistant to geometric effects. Based on the behavior of spindles in *par-3* mutant embryos, it has been proposed that PAR-3 stabilizes interactions between the astral microtubules and the cell cortex (Etemad-Moghadam et al., 1995; Grill et al., 2001), although the mechanism is unknown. Such a role might be a clue for how PAR-3 functions, directly or indirectly, to resist the effects of geometry in nonpolarized cells as well. To understand more about the novel function of PAR-3 in inhibiting geometry-dependent rotation, we looked for potential candidates that could act downstream of PAR-3. Of the spindle orientation genes described in the literature, only LET-99 has been shown to be localized and/or regulated by PAR-3 (Tsou et al., 2002). LET-99 is uniformly localized around the cortex in *par-3* mutants, and the level of cortical LET-99 is higher compared with the levels of cortical LET-99 in wild-type AB cells and *par-2* mutant cells (Tsou et al., 2002), where geometry-dependent rotation does not occur. To determine if this ectopic high level of LET-99 is required for *par-3* cells to be sensitive to geometric asymmetry, we traced centrosome movements in *par-3:let-99* double RNAi embryos. In contrast to the directed rotation toward the cell contact region observed in *par-3* embryos, nuclear–centrosome complexes in *par-3;let-99* embryos exhibited a random hyperactive rocking motion similar to that described for *let-99* embryos (Fig. 4, A–F). In some cases, the nuclear–centrosome complex changed 90° in orientation in only 10–20 s, and the direction of change appeared completely random (Fig. 4 G). These results indicate that LET-99 is required for *par-3* cells to be sensitive to geometric asymmetry, and suggest that having low levels instead of high levels of cortical LET-99 in wild-type AB cells allows them to resist the effects of cell shape.



G

Initial \ Final	A/P	D/V	L/R
A/P n=5	3	0	2
D/V n=14	8	3	3
L/R n=11	5	5	1

Figure 4. ***let-99* is required for *par-3* embryos to be sensitive to geometric effects.** (A–F) Time-lapse series of a two-cell *par-3;let-99* double RNAi embryo. The centrosomes were visualized with GFP-tubulin (A–E) and then the final division pattern shown by DIC microscopy (F). In this example, hyperactive centrosome movements were observed in which the nuclear–centrosome complex in the posterior cell moved from a 45° angle (B) to longitudinal (C) then to a left/right orientation (D and E). (G) Quantification of changes in nuclear–centrosome positioning in *par-3;let-99* double RNAi embryos. The initial and final position of the centrosomes were scored after centrosomes were separated and during nuclear envelope breakdown respectively. A/P, anterior/posterior; D/V, dorsal/ventral; L/R, left/right; n, total number in that category. All *par-3;let-99* double RNAi embryos exhibited a symmetric first cleavage and synchronous second cleavage, indicating that the *par-3* RNAi was effective. Bar, 10 μm.

Discussion

Polarity-dependent and geometry-dependent mechanisms of spindle orientation

In this paper, we have investigated the role played by cell shape in the nuclear rotation events that orient the mitotic spindle in *C. elegans* embryos. Our data show that centration and nuclear rotation do not depend on cell shape. However, the close juxtaposition of the nucleus to the cell contact region after rotation in wild-type P_1 cells is cell shape-dependent. In wild-type embryos where the P_1 cell is made spherical, nuclear rotation still occurs. However, such rotation is a free rotation where the nucleus remains centrally located, instead of a directed rotation toward the cell division remnant.

These results challenge the prevailing model for nuclear rotation in *C. elegans* embryos. The cortical site model proposes that an anterior cortical site captures and shortens microtubules, thus causing nuclear rotation directed toward the anterior cortex of P_1 cell (Hyman and White, 1987; Hyman, 1989). This model was first developed based on several

lines of evidence. First, during centration and rotation, the nucleus in the P_1 cell moves toward the cell contact region, and after rotation, the nucleus becomes closely juxtaposed to the anterior cortex. Second, ablation of the cytoplasm in the area between the centrosomes and the anterior cortex causes rotation to cease; ablation of lateral or lateral posterior areas does not stop rotation. Third, the membrane at the cell contact region invaginates into the P_1 cell during rotation, suggesting tension on that part of the anterior cortex. Subsequently, it was found that some components of the dynein associated dynactin complex accumulate transiently in a dot at the anterior cortex (Waddle et al., 1994; Skop and White, 1998), and the model has evolved into one in which the anterior cortical site that captures microtubules is localized at or around the cell division remnant (Waddle et al., 1994; Keating and White, 1998; Skop and White, 1998; Gönczy, 2002). Although dynein and dynactin are required for rotation in the one-cell embryo, it has not been possible to directly test their roles in P_1 (Skop and White, 1998; Gönczy et al., 1999). In addition, it has not been shown whether the dot of dynactin or the membrane invagination are a cause or an effect of rotation.

The free rotation that we observed in P_1 spherical cells cannot be explained by the presence of a localized cortical site that captures and shortens microtubules because that model predicts the nucleus will move toward the cortical site even in a spherical cell (Fig. 1 A; Hyman, 1989). In addition, we have found that nuclear rotation in *par-3* mutant embryos, which lack intrinsic polarity, does not occur when extrinsic cell shape asymmetry is removed. However, nuclear rotation can be induced by introducing ectopic asymmetry via a flat side. Thus, although nuclear rotation in *par-3* embryos was previously considered as evidence for ectopic activation of the cortical site in the AB cell, our results clearly indicate that rotation in *par-3* embryos is driven by cell shape. Our results also indicate that the membrane invagination associated with the division remnant correlates with a specific cell shape and is not required for centration or nuclear rotation. The observations that the membrane invagination are visible in the irradiated cell in both *par-3* and wild-type embryos, and that astral microtubules respond to other cortical cues that position the spindle in the unirradiated spherical cell suggest that UV irradiation has not damaged the cortex. However, whether the cortical site is still present in wild-type spherical P_1 cells does not appear relevant. If the cortical site is there, it is not acting as predicted by the model. If the site has been damaged, then the cortical site is not essential because rotation still occurs. Therefore, we conclude that the combination of findings on cell shape effects in both wild-type and *par-3* embryos are incompatible with the specialized cortical site model for nuclear rotation.

The occurrence of free rotation in spherical cells suggests that another type of mechanism functions in P_1 cells. We propose that nuclear rotation in wild-type one- and two-cell embryos is driven by a programmed polarity-dependent mechanism that causes free rotation, and that the extended movement of the nucleus toward the anterior cortex in P_1 cells, as well as the localized invagination of the cortex, are consequences of the physical effects of cell shape. LET-99 is likely to be involved in the polarity-dependent mechanism of rotation because LET-99 is required for nuclear rotation in both P_0 and P_1, LET-99 is localized in response to PAR-3, and the LET-99 band model predicts free central rotation in spherical cells. This model is consistent with the original laser ablation studies that led to the anterior cortical site model (Hyman, 1989) because the combination of the LET-99 band and cell shape is predicted to result in higher pulling forces directed toward the anterior cortex.

The results of these cell shape studies also significantly change the interpretation of how the PAR proteins control nuclear rotation in wild-type embryos. In contrast to the original model, the findings reported here indicate that PAR-3 and PAR-2 are both required for normal nuclear rotation. Importantly, PAR-3 does not inhibit normal nuclear rotation, but does inhibit geometry-dependent rotation. Our findings also explain previous reports that flattening *par-3* embryos resulted in a lower frequency of rotation toward the cell contact region (Cheng et al., 1995). The ectopic flat side of the coverslip would be predicted to compete with the flat cell contact region in directing rotation in such embryos.

A physical model for geometry-dependent nuclear rotation in *par-3* embryos

Although the molecular mechanism remains to be determined, it is intriguing that the geometry of two different cell shapes, that of oval cells and cells with one flat/curved-in side, can cause nuclear rotation in *par-3* cells. To understand how geometry might work in driving nuclear rotation in cells with no intrinsic polarity, we used several known microtubule properties as assumptions to build a physical model. The first assumption is that microtubules are laterally associated with motors on the cortex, such as dynein, rather than associated end-on. It has been reported that microtubules can interact with the cell cortex either end-on or laterally (Adames and Cooper, 2000). With end-on interactions, microtubule plus ends reach the cortex without bending and are captured by cortical proteins. By depolymerization of microtubules, motile forces can be generated to move objects (Lombillo et al., 1995). The magnitude of forces generated on each microtubule with end-on attachments depends largely on the rate of depolymerization, and will be independent of the angles with which microtubules interact with the cell cortex. Therefore, if the depolymerization rate is uniform around the cortex as predicted for nonpolarized *par-3* cells, every microtubule will experience a similar amount of force regardless of the cell shape. With this scenario, geometric asymmetry should have no effect on nuclear rotation and centrosome movements in *par-3* embryos. Conversely, if microtubules interact laterally with the cortex, the interaction angle will significantly affect the force vector applied on each microtubule (Fig. 5 A). Therefore, our model is based on lateral attachments. The second assumption is that microtubules tend to bend at small angles due to their stiffness (Gittes et al., 1993).

Based on these assumptions, microtubules that contact the cortex with small angles will experience a greater force vector toward the cortex (and thus produce greater pulling on the nuclear–centrosome complex) than those that contact the cortex at angles near 90° (Fig. 5, A1–A3). Microtubules as-

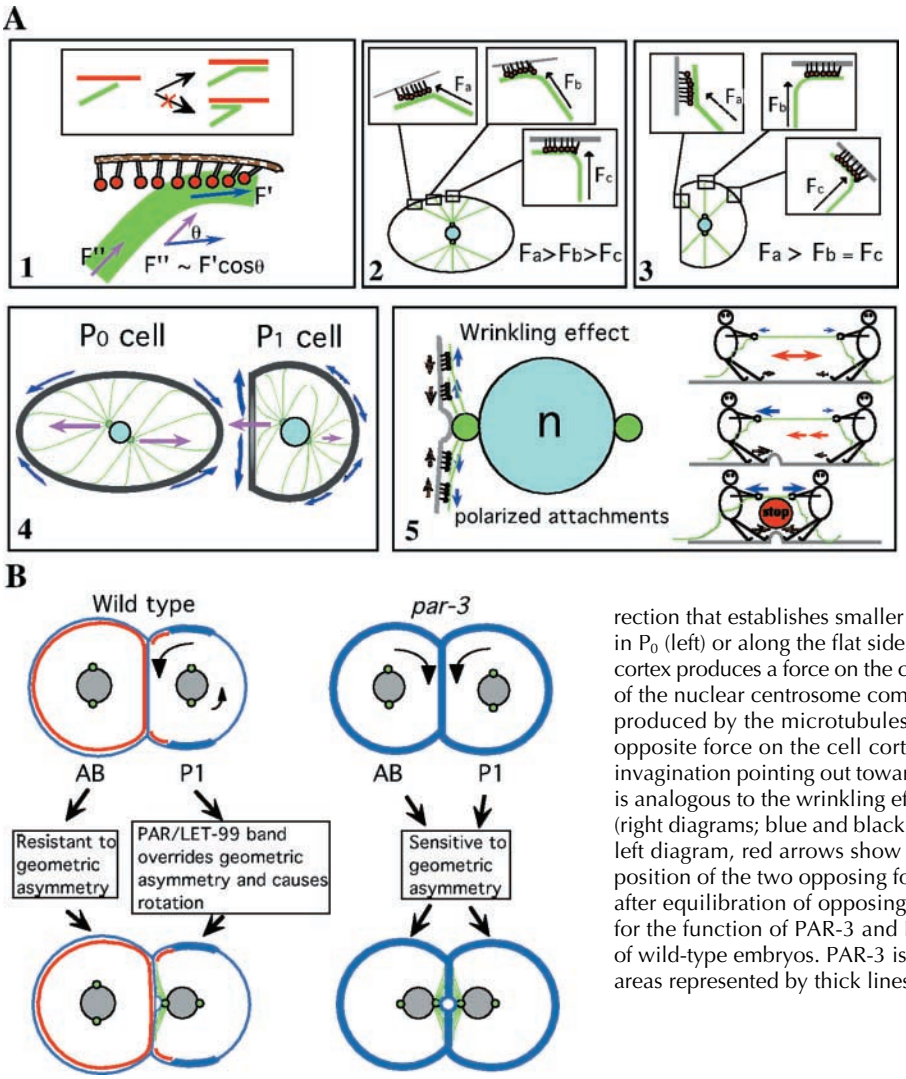


Figure 5. **Models for geometry-dependent and PAR-dependent nuclear rotation.** (A) A physical model for geometric effects on nuclear movements in the absence of polarity in *par-3* mutant embryos. Microtubules are shown in green, motor molecules in red, and nuclei (n) in blue. (1) Microtubules in lateral association with cortex. The force vector toward the cortex (F′′, purple arrows) equals the sliding force (F′, blue arrows) times cosine of the angle with the cortex (θ). Inset shows bending of microtubules; stiffness prevents microtubules from associating with angles larger than 90°. (2 and 3) Microtubules that contact the cortex with small angles will experience greater force vectors toward the cortex. Note that in *par-3* one-cell embryos, the pronuclei meet in the center. (4) Tilting of the nuclear–centrosome complex by dynamic perturbation will lead to sliding (blue arrows) of microtubules in the direction that establishes smaller association angles, that is toward the poles in P_0 (left) or along the flat side in P_1 (right). Sliding of microtubules on the cortex produces a force on the centrosomes (purple arrows). (5) Final position of the nuclear centrosome complex in a flat-sided cell. The polarized force produced by the microtubules (blue arrows) is predicted to produce an opposite force on the cell cortex (black arrows) that causes a localized invagination pointing out toward the centrosome. This proposed interaction is analogous to the wrinkling effect on a carpet during a game of tug-of-war (right diagrams; blue and black arrows are analogous to forces shown in the left diagram, red arrows show movements or fluctuations of the central position of the two opposing forces, and "stop" indicates the final position after equilibration of opposing forces; see text for explanation). (B) Model for the function of PAR-3 and LET-99 in polarized and nonpolarized cells of wild-type embryos. PAR-3 is shown in red, LET-99 in blue with enriched areas represented by thick lines.

sociated with the cortex at 90° will have a near-zero force vector toward the cortex, and thus will slide more actively along the cortex. If the cell is not spherical, sliding in a direction where smaller association angles are established will be favored, at which point the force vector toward the cortex will increase, which can in turn move the nuclear–centrosome complex. Thus, in nonspherical cells, when the centrosomes are aligned perfectly perpendicular to the anterior/posterior axis, the forces on the microtubules will be balanced and the nuclear–centrosome complex will temporarily remain stationary (Fig. 5 A2). However, this is an unstable steady state, and any stochastic perturbation that slightly tilts the nuclear–centrosome complex will result in a disruption of the balance and shifting of the nuclear–centrosome complex from the initial position toward an orientation where a stable steady-state is established (Fig. 5 A4). In particular, in oval-shaped *par-3* cells like P_0, the astral microtubules from each of the two centrosomes will always slide toward the two poles of the long axis (Fig. 5 A4 left, blue arrows). As a result, one centrosome will be pulled toward the anterior, whereas the other will be pulled toward the posterior, resulting in free nuclear rotation without net movement of the nucleus past the center point of the long axis. Orientation on the long axis will be the stable orientation. In *par-3* cells with one flat side, as seen in two-cell embryos, microtubules that interact with the flat cortex will slide along the surface until they reach smaller angles. Thus, in cross-sectional view (Fig. 5 A5), microtubules will split into two groups that move away from each other (Fig. 5 A5 right, blue arrows), which in turn will pull the centrosome toward the flat cortex until it is tightly associated with the cell contact (Fig. 5 A5 left), causing rotation. Thus, rotation directed toward the cell contact region is a predicted consequence of the astral microtubules sliding apart on a flat surface. Such an interaction is predicted to cause nuclear rotation in *par-3* embryos, and could also explain the geometry-dependent juxtaposition of the nucleus to the anterior cortex in wild-type P_1 cells.

Another predicted outcome of the model for cells with one flat side is that the polarized microtubule attachments with dynein will produce an opposite force on the membrane, causing a localized invagination of the cortex at the cell contact region in both *par-3* and wild-type P_1 cells (Wrinkling effect, Fig. 5 A5), analogous to the wrinkling up of a carpet in a game of tug-of-war. Physically, this wrinkling effect should only occur where the two membranes are

not as tightly sealed, such as at the gap present at the cell division remnant (Keating and White, 1998; Skop et al., 2001). Membrane invaginations adjacent to the cell division remnant were previously considered as evidence for the cortical site model in both *par-3* and wild-type embryos (Hyman and White, 1987; Hyman, 1989; Waddle et al., 1994; Keating and White, 1998). However, our physical model suggests that invagination could be a consequence rather than a cause of nuclear rotation. The absence of the invaginations in wild-type spherical P_1 cells supports this view. Furthermore, the model can also explain why the centrosome is always positioned at the division remnant (the site of the invagination) after nuclear rotation has occurred. In analogy to the tug-of-war game (Fig. 5 A5), if the magnitudes of two dynamically opposing forces are relatively similar to each other, the central position between the two will randomly fluctuate along the surface of the carpet (Fig. 5 A5, top). However, the appearance of a wrinkle on the carpet will dramatically limit the extent of the fluctuation and thus settle and fix the final central position between the two around the wrinkled-up region on the carpet (Fig. 5 A5, middle and bottom). Live imaging of centrosome movements in wild-type embryos has documented such "overshooting" and equilibration to a final position adjacent to the division remnant (Keating and White, 1998). However, in spherical P_1 cells, we did not observe rotation oriented toward the division remnant, consistent with the model that the close association of the centrosome in unaltered P_1 cells is a consequence of cell shape. The biological meaning for this cell shape-dependent effect of the cell division remnant in fixing the final position of the centrosome is unknown, but our results clearly show that the invagination itself is not required for nuclear rotation and asymmetric spindle elongation in wild-type P_1 cells.

PAR-3 regulates spindle orientation in both polarized and nonpolarized cells, potentially through LET-99

In this paper, we have shown that extrinsic geometry can drive ectopic nuclear rotation, which would appear to be problematic for normal development. Polarized cells of the wild-type *C. elegans* embryo avoid this problem through a PAR polarity–dependent mechanism that can override geometric effects and drive wild-type rotation. However, the nonpolarized wild-type AB cell is also resistant to geometric effects and does not show ectopic spindle alignment toward the cell contact region. *par-3* mutants are sensitive to geometric affects, indicating that PAR-3 is also required to suppress ectopic geometry-driven rotation in nonpolarized cells. The underlying mechanisms for both may rely on PAR-3's role in LET-99 localization. The localization of LET-99 to a band in the P cells requires PAR-3 and PAR-2, and the absence of the band correlates with failure of nuclear rotation in *par-3* and *par-2* embryos (Tsou et al., 2002; this paper). In addition, high levels of PAR-3 appear to inhibit localization of LET-99 at the anterior cortex in P cells and at the cortex in AB cells (Tsou et al., 2002). In wild-type AB cells and *par-2* mutant two-cell embryos, where PAR-3 is uniformly present and LET-99 is uniformly low, nuclear–centrosome complexes do not orient toward the cell contact region. However, in *par-3* embryos where geometry-dependent rotation occurs, LET-99 is present at higher levels at the cortex (Cheng et al., 1995; Tsou et al., 2002; this paper). Furthermore, LET-99 is required in *par-3* embryos for the ectopic alignment according to geometry. Although it is possible that loss of LET-99 prevents geometry dependent rotation in *par-3*;*let-99* embryos indirectly, the correlations between PAR-3 levels, LET-99 levels and resistance to geometry are striking. Therefore, we favor the hypothesis that in wild-type embryos, PAR-3 suppresses the effects of cell shape by down-regulating the levels of cortical LET-99 in the AB cell. We propose that it is not only critical that LET-99 be present in a band in cells that undergo nuclear rotation onto the polarized axis, but also for LET-99 levels to be low, but not absent, in other cells to prevent geometry-driven rotation (Fig. 5 B).

In summary, we have found that PAR-3 is essential for the mechanism of nuclear rotation in asymmetrically dividing cells, as well as for avoiding ectopic rotation in nonpolarized cells. The presence of PAR-3 in both types of cells provides a way of regulating the division pattern in multiple cells as a response to embryonic polarity. Such regulation is likely important in other organisms during development, where cells are dividing in multicellular contexts and cell shapes are not spherical. The effects of geometry would need to be modulated in order to produce the normal patterns of division necessary for segregation of cell fate determinants and for morphogenesis.

Materials and methods

Strains and maintenance

C. elegans were cultured using standard conditions (Brenner, 1974). Strains used in this work were as follows: N2, wild type Bristol; WH204, *pie-1::GFP::β-tubulin* (Strome et al., 2001); KK653, *par-3(it71) unc-32(e189)/qC1*. Strains were provided by the Caenorhabditis Genetics Center (N2; University of Minnesota, Minneapolis, MN), the K. Kemphues lab (KK653; Cornell University, Ithaca, NY), and the J. White lab (WH204; University of Wisconsin, Madison, WI). All worms were grown at 20°C and filmed at 23–25°C. The *par-3 (it71)* allele was used for all analyses except imaging of GFP-labeled tubulin.

RNA interference

Antisense and sense RNAs were transcribed in vitro (MEGAscript™; Ambion) from linear DNA templates. Templates were produced by the polymerase chain reaction, using T3 and T7 primers to amplify a partial *par-3* cDNA (a gift from K. Kemphues), *par-2* cDNA (a gift from L. Boyd; University of Alabama, Huntsville, AL), or a full-length *let-99* cDNA. Double-stranded RNAs (1 mg/ml^{-1}; Fire et al., 1998) were injected into adult hermaphrodites, and progeny were analyzed 16–24 h later. *par-3;par-2* double-mutant embryos were generated by soaking *par-3(it71)* hermaphrodites in 1.5 mg/ml^{-1} *par-2* dsRNA (Tabara et al., 1998). Wild-type hermaphrodites were simultaneously soaked and examined as a control for effectiveness of *par-2(RNAi)*.

Microscopy and alteration of cell shape

Embryos were mounted unflattened (Rose and Kemphues, 1998a) or flattened with 22 × 22-mm or 22 × 30-mm coverslips on a thin pad of 5% agarose, and examined under differential interference contrast (DIC) or fluorescence optics using time-lapse microscopy. To produce spherical P_1 cells in early prophase two-cell embryos, the field diaphragm was closed down to restrict the beam of light to the anterior half of the AB blastomere; the field was then irradiated with UV light (330–385 nm) from a 100-W mercury lamp for 3–7 s. Only those embryos in which P_1 was unaffected by irradiation, as judged by normal cell cycle length, were analyzed further. To confirm that mounting embryos on agar pads under coverslips induces an ectopic flat side, a complete Z series (1-μm step) for the top and bottom halves of the same embryo were collected separately on an upright and inverted microscope, respectively (due to the limited working distance

of the objective). Images were then manually examined to determine the in-focus area as judged by the appearance of yolk granules. The in-focus information was then used to generate three-dimensional reconstructions with MetaMorph® software (Universal Imaging Corporation).

Online supplemental material

Fig. S1 documents the spherical and flattened shapes of cells. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb200209079/DC1.

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