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The *let-99* gene is required for proper spindle orientation during cleavage of the *C. elegans* embryo

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SUMMARY

The orientation of cell division is a critical aspect of development. In 2-cell *C. elegans* embryos, the spindle in the posterior cell is aligned along the long axis of the embryo and contributes to the unequal partitioning of cytoplasm, while the spindle in the anterior cell is oriented transverse to the long axis. Differing spindle alignments arise from blastomere-specific rotations of the nuclear-centrosome complex at prophase. We have found that mutations in the maternally expressed gene *let-99* affect spindle orientation in all cells during the first three cleavages. During these divisions, the nuclear-centrosome complex appears unstable in position. In addition, in almost half of the mutant embryos, there are reversals of the normal pattern of spindle orientations at second cleavage: the spindle of the anterior cell is aligned with the long axis of the embryo and nuclear rotation fails in the posterior cell causing the spindle to form transverse to the long axis. In most of the remaining embryos, spindles in both cells are transverse at second cleavage. The distributions of several asymmetrically localized proteins, including P granules and PAR-3, are normal in early *let-99* embryos, but are perturbed by the abnormal cell division orientations at second cleavage. The accumulation of actin and actin capping protein, which marks the site involved in nuclear rotation in 2-cell wild-type embryos, is abnormal but is not reversed in *let-99* mutant embryos. Based on these data, we conclude that *let-99(+)* is required for the proper orientation of spindles after the establishment of polarity, and we postulate that *let-99(+)* plays a role in interactions between the astral microtubules and the cortical cytoskeleton.

Key words: Spindle orientation, Nuclear rotation, Asymmetric division, *Caenorhabditis elegans*, *let-99*, Cleavage

INTRODUCTION

In organisms ranging from yeast to mammals, certain cells have reproducible patterns of division during growth and development (Kropf, 1992; Jürgens, 1995; Rhyu and Knoblich, 1995; Chant, 1996; White and Strome, 1996). The timing and orientation of division contributes to the spatial organization of cells and thus can be important for morphogenesis, tissue organization and cellular interactions that influence developmental fate (Mello et al., 1994; Reinsch and Karsenti, 1994; Torres-Ruiz and Jürgens, 1994; Smith et al., 1996). Division orientation also plays a role in the segregation of cellular components during intrinsically asymmetric divisions (Strome and Wood, 1983; Chenn and McConnell, 1995; Kraut et al., 1996). In animal cells, division orientation is determined by the position of the mitotic spindle: the cleavage furrow always bisects the spindle (Salmon, 1989). Studies of oriented divisions in invertebrates suggest that astral microtubules interact with the cell cortex to position the spindle (Hyman and White, 1987; Schroeder, 1987; Hyman, 1989; Kawamura and Yamashiki, 1992; McGrail and Hays, 1997). The dependence of spindle position on an intact actin and/or microtubule cytoskeleton has also been demonstrated in algae and budding yeast (e.g. Snyder et al., 1991; Kropf, 1992; Palmer et al., 1992).

The nematode *Caenorhabditis elegans* is an excellent system for studying mechanisms underlying cell division patterning in a multicellular organism. The pattern of cell division is virtually invariant from animal to animal, and the transparency of the organism facilitates examination of division pattern in living specimens (Sulston et al., 1983). The embryonic division pattern is characterized by a series of unequal divisions. During the division of the one-cell, P_0, the spindle is aligned along the longitudinal (anterior-posterior) axis of the embryo and becomes positioned closer to the posterior pole. First cleavage is therefore unequal, giving rise to a larger anterior cell, AB and a smaller posterior cell, P_1. At second cleavage, the P_1 spindle is oriented on the same axis as the first spindle. At third cleavage, similar orientations are seen in the daughters of P_1 (EMS and P_2). In both cells, the spindle pole is oriented towards the membrane furrow that separates EMS and P_2; the EMS spindle is thus anterior-posterior, but the P_2 spindle is dorsal-ventral due to the cellular arrangements within the eggshell. The divisions of P_1, EMS and P_2 are

asymmetric in terms of cell size, the inheritance of cytoplasmic factors and cell fate potential (Laufer et al., 1980; Strome and Wood, 1983). For example, only the P cells inherit P granules. In contrast, the spindle axes of AB and its daughters are perpendicular (transverse) to the previous axes and the divisions are symmetric.

The two patterns of spindle position at second cleavage (longitudinal and transverse) result from differences in centrosome movement (Hyman and White, 1987; Hyman, 1989). In the P_1 cell, there is a 90° rotation of the nuclear-centrosome complex during prophase so that the spindle aligns on the longitudinal axis once again. Rotation is dependent on an interaction between the astral microtubules and an anterior cortical site and also requires actin filaments. Waddle et al. (1994) have shown that actin and actin capping protein transiently localize to the anterior cortex, consistent with a role for these proteins in nuclear rotation. Similar rotations occur in the 1-cell (P_0) and in the daughters of the P_1 cell (EMS and P_2).

Studies of the *par* genes indicate that the difference in cleavage pattern between the AB and P_1 cells is dependent on the establishment of polarity in the embryo (Kemphues et al., 1988). Mutations in the *par* genes perturb many of the asymmetries normally present in the 2-cell embryo, such as the posterior localization of P granules and the different cell fate potentials of AB and P_1. Some *par* mutations also alter spindle orientations at second cleavage. In particular, genetic and molecular analyses indicate that the PAR-3 protein plays an important role in preventing nuclear rotation in the AB cell (Cheng et al., 1995; Etemad-Moghadam et al., 1995). It is unclear whether PAR-3 functions directly or indirectly in preventing nuclear rotation, or how many other factors are involved in controlling the difference in pattern between the AB and P lineages. One such factor may be the G protein β subunit encoded by the *gpb-1* gene, which is required for normal spindle orientations in several cells during early cleavage (Zwaal et al., 1996). Thus, while several proteins required for normal spindle orientation have been identified, the precise mechanism of nuclear rotation and its regulation are unknown.

We have taken a genetic approach to identify additional molecules required for orienting spindles in *C. elegans* embryos. We have isolated several mutations that cause alterations in the early cleavage pattern and here present a characterization of mutations in the *let-99* gene. Our observations suggest that the *let-99* gene is required for normal spindle orientation in both the AB and P1 cells, and *let-99* mutants can show a reversal of the normal pattern of spindle orientations. However, other aspects of asymmetry are normal in *let-99* mutant embryos, suggesting that *let-99* functions downstream of the *par* genes and the establishment of polarity in the control of spindle orientation.

MATERIALS AND METHODS

Strains

C. elegans strains were cultured as described in Brenner (1974). Strains containing the following mutations or rearrangements were used: LGIV: *him-03(e1147), egl-23(n601), dpy-4(e1166), dpy-20(e1282ts), unc-5(e152), unc-22(e66), unc-22(s7), unc-31(e169), eDf19, sDf2, sDf21, sDf22, sDf62*; *nT1[unc(n754) let] IV;V.* LGIII**:** *par-2(lw32), unc-45(e286ts), sC1 [dpy-1(e1) let], sma-4(e729), par-3(it71), qC1*. Bristol N2 or *unc-22(e66)* worms were used as wild-type *(let-99+)* controls. Strains were obtained from the Caenorhabditis Genetics Center or constructed in the Kemphues laboratory. For all experiments, worms were grown at 20°C, but during videomicroscopy, embryos were at 23–25°C. There were no differences in the phenotypes of *let-99* mutants grown at 16°, 20° or 25°C (Table 1).

Genetics

Maternal effect lethal (mel) mutations were isolated using the procedure outlined in Kemphues et al. (1988), using *egl-23 him-3* hermaphrodites mutagenized with 50 mM EMS. From 3317 F_1 individuals, 508 lines that segregated F_2 worms bearing mel mutations were isolated. 221 lines, those in which embryos arrested with large numbers of cells but abnormal morphogenesis, were further analyzed by videomicroscopy for cleavage pattern defects in the embryos. 15 mutations that produced cleavage pattern phenotypes were isolated, including the *it141* mutation. The *it141* mutation was mapped to chromosome IV using Sequence Tag Site mapping (Williams et al., 1992) and was mapped by meiotic recombination to the *unc-22 dpy-4* interval. The deletions *eDf19* and *sDf62* complemented *it141*, while *sDf2, sDf21* and *sDf22* failed to complement. The *let-99(s1201*) mutation, which maps to the *sDf2 sDf22* overlap (Clark et al., 1988), failed to complement *it141* for maternal effect lethality. *it141* was outcrossed five times in the process of constructing *unc-22(e66) let-99(it141)/nT1 [unc(n754) let] IV;V* which was the source of *let-99(it141)* mutants for all experiments. *unc-5 unc-22(s7) let-99(s1201) unc-31/nT1 IV;V* was outcrossed five times to construct an *unc-22(s7) let-99(s1201)/nT1 [unc(n754) let] IV;V* strain which was used for experiments after the initial complementation test.

The *let-99* alleles behave as maternal effect mutations. Virtually all embryos from *let-99* mutant mothers fail to hatch (Table 1). A wild-type copy of *let-99* contributed by male sperm does not rescue the embryonic lethality; 0/192 embryos hatched when *unc-22 let-99(it141)* hermaphrodites were sperm depleted and then mated to N2 males (3 crosses). In addition, virtually all of the embryos from *let-99*/+ hermaphrodites hatched (519/522 for *unc-22 let-99 (it141)*, 499/509 for *unc-22 let-99(s1201)*) suggesting that there is no zygotic effect of this mutation on embryonic viability. The recovery of *unc-22* adults from these heterozygous mothers was slightly lower than expected (20.6% for *unc-22 let-99(it141), n*=519; 19.6% for *unc-22 let-99(s1201), n*=499; 26.4% for *unc-22, n*=284). Sperm-depleted N2 hermaphrodites mated to *dpy-20 let-99(it141)* males produced almost all viable progeny (589 males, 608 hermaphrodites, 7 unhatched), indicating that there is no paternal effect of *let-99(it141)* on embryonic viability.

Microscopy

Embryos were cut from hermaphrodites and filmed under Nomarski optics using a time-lapse videorecorder and CCD camera. For most observations, embryos were mounted on poly-lysine-coated coverslips in water, then inverted over a slide using additional coverslips as spacers to prevent flattening. The measurements in Table 2 were made from monitor tracings of videotaped embryos. Nuclear-centrosome and spindle orientations were scored from late prophase through metaphase; after this time, cell shape changes can obscure and distort the spindle orientation. For second cleavage, divisions were scored as longitudinal if the spindle was aligned within 0–45° of the long axis of the egg. Spindles at angles greater than 45° were scored as transverse. For third cleavage, orientation was scored with respect to the previous division's spindle orientation and the resulting division furrow, since cell positions change relative to the longitudinal axis in both wild-type and *let-99* embryos. For some observations, embryos were 'flattened' by mounting on 5% agar pads under a coverslip.

Laser ablations

To determine the *let-99* terminal phenotype, intact embryos were incubated on poly-lysine-coated slides at 20°C in a moist chamber until the time of wild-type hatching. For cell ablations, embryos were mounted on agar pads and viewed under Nomarski optics at 100× magnification. A VSL-337 nitrogen laser (Laser Science, Inc.) was used to irradiate nuclei with approximately 50-100 pulses of light until significant debris formed within the nucleus, as described in Mello et al. (1992). After ablation, partial embryos were allowed to develop at 20°C for 16-20 hours in a moist chamber. Embryos were examined for differentiated cell types or tissues using Nomarski or polarization optics as described in Bowerman et al. (1992) and Mello et al. (1992).

Immunofluorescence

Embryos were fixed and stained for immunofluorescence using anti-P granule antibody OIC1D4 or K76, following the protocol of Strome and Wood (1983; provided by S. Strome or the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; contract N01-HD-2-3144, NICHD). Immunolocalization of tubulin, PAR-1, PAR-2 and PAR-3 were performed as described in Kemphues et al. (1986), Guo et al. (1995), Boyd et al. (1996) and Etemad-Moghadam et al. (1995), respectively. For all immunofluorescence experiments, embryos were also stained with 4′,6 diamidino-2-phenylindole dihydrochloride (DAPI) to visualize nuclei. Method II of Waddle et al. (1994) was used for anti-CP and anti-actin (monoclonal C4, ICN) staining, since it allows for fixation of small numbers of mutant embryos attached to poly-lysine slides. CP and actin accumulations appeared smaller using this method than with Method I and, in about 50% of embryos (both wild type and *let-99*), the accumulation was associated with a gap between the AB and P1 cells. In addition, the size of dots and plaques varied from one preparation to another, even in wild type, and so *let-99* and wild-type embryos were always prepared in parallel. Division orientation in actin/CP-stained embryos was determined by double staining with anti-tubulin antibodies and/or DAPI. Specimens were viewed and photographed using a Zeiss or Olympus Photomicroscope.

RESULTS

Identification of mutations that alter cleavage pattern

In order to identify genes involved in controlling the orientation of cell division in *C. elegans*, we generated a collection of maternal effect lethal mutants. Embryos from homozygous mutant mothers were observed during the first few cleavage divisions using time-lapse videomicroscopy. We isolated 10 mutations that produced altered cleavage patterns (excluding alleles of previously known genes such as the *pars*). The characterization of one of these mutants, *it141*, is presented here. We mapped the *it141* mutation to chromosome IV and found that it failed to complement *let-99(s1201*) (Clark et al., 1988). Embryos from homozygous *let-99(s1201)* mothers show the same phenotype as embryos from *let-99(it141)* mothers. Both mutations act as recessive maternal effect lethal mutations (see Methods) and show the same phenotype in *trans* to a deletion for the region (Tables 1–3). Thus, we believe that *it141* and *s1201* represent strong loss-of-function mutations in the *let-99* gene. The two alleles are virtually indistinguishable in terms of maternal effect lethality and cleavage pattern phenotype (Tables 1–3). The observations presented in this report are based on observations of *it141* unless otherwise noted. For simplicity, we will refer to embryos from homozygous mutant mothers as *let-99* embryos.

Table 1. Maternal effect lethality of *let-99* mutations

Genotype	Temperature °C	Number of eggs laid	% Hatch
wild type*	20	1828	96.9
*let-99(it141)**	16	2174	0.87
	20	3105	0.39
	25	1049	0.48
let-99(it141)/sDf22	20	4452	0.20
let-99(s1201)†	16	1414	0.35
	20	3573	0.36
	25	ND‡	ND‡
let-99(s1201)/ sDf22	20	4025	0.50

Eight to ten hermaphrodites of each genotype were put individually on plates and transferred daily. The number of eggs laid and whether they hatched was scored for the entire brood of each hermaphrodite.

*Marked with *unc-22(e66).*

†Marked with *unc-22(s7)*.

‡ND, not determined because *unc-22(s7)* hermaphrodites are egg-laying defective at 25°C.

let-99 is required for the normal pattern of early cleavages

To characterize the role of the *let-99* gene in spindle orientation, we examined *let-99* mutant embryos by time-lapse videomicroscopy and anti-tubulin immunofluorescence. Defects in nuclear positioning and spindle orientation during the first three cleavages were observed. The microtubule networks in *let-99* embryos were otherwise indistinguishable from wild-type.

Spindle orientation defects

The first cell cycle events of *let-99* and wild-type embryos are summarized schematically in Fig. 1 and quantified in Table 2. In wild-type embryos, the female pronucleus migrates to meet the male pronucleus in the posterior of the embryo. The pronuclear-centrosome complex then moves to the center of the embryo and rotates 90° (usually before nuclear envelope breakdown), resulting in mitotic spindle formation on the longitudinal axis of the embryo. The spindle moves and elongates asymmetrically in the posterior direction, and first cleavage is unequal. In *let-99* mutant embryos, the pronuclei met in the posterior (Fig. 1F), but failed to move completely to the center of the embryo. In addition, in many embryos, the nucleus did not complete rotation before nuclear envelope breakdown, and the spindle formed at an angle (compare Fig. 1B and G; Table 2). In most embryos, as the misoriented spindle elongated it moved within the cytoplasm onto the correct axis (Fig. 1H). Thus, first cleavage in *let-99* embryos produced a 2-cell stage resembling wild type (Fig. 2A,E) except that the cells were slightly more unequal in *let-99* embryos (Table 2).

In wild-type embryos at second cleavage, the AB spindle is transverse while the P1 nucleus undergoes rotation and the spindle is longitudinal. At second cleavage in *let-99* embryos, abnormal spindle orientations were observed in both the AB and P_1 cells; Table 3 tabulates the different patterns. In approximately half of the mutant embryos, the AB spindle was normal, but in half it was longitudinal (compare Fig. 1D,I). Intermediate positions of the nuclear-centrosome complex were seen in prophase in *let-99* mutants, especially in AB, but these tended to resolve into either transverse or longitudinal

Table 2. First cell cycle phenotypes of *let-99* mutants

Genotype	*n*	Position of pronuclear meeting*	Position of pronuclei at mitosis onset†	Angle of spindle at mitosis onset, <30°	30-60°	>60°‡	Position of cleavage furrow§
wild type	10	68.3±3.7	50.3±2.1	9	1	0	53.9±1.7
let-99(it141)	18	66.9±2.5	61.0±3.1	9	6	3	57.0±2.1
let-99(it141) / sDf22	10	65.3±2.6	63.5±1.8	0	4	6	56.2±2.1
let-99(s1201)	12	67.2±2.5	62.3±2.4	4	6	2	56.8±2.4
let-99(s1201)/ sDf22	13	66.7±3.8	64.1±2.4	2	8	3	56.5±2.7

Positions are expressed as percentage of egg length (mean ± standard deviation) with anterior equal to 0%. *n*=number of embryos.
*Position of mid point between the pronuclei at meeting.
†Position of mid point between the pronuclei, just after nuclear envelope breakdown.
‡Angle of the axis defined by a line drawn through the two centrosomes at the time of pronuclear envelope breakdown. 0°=longitudinal (aligned with anterior-posterior axis); 90°=transverse to that axis. The number of embryos in each class is shown. In most *let-99* embryos, the spindle moved through the cytoplasm onto the correct axis even if it formed at an angle. In a small number of embryos where the spindle set up transversely (for example 2/18 *it141* embryos above) the entire embryo rotated within the eggshell until the spindle was on the longitudinal axis. As a result, the posterior pole of the embryo assumed a lateral position, and cleavage was equal. Those embryos divided synchronously at second cleavage.
§Position of the cytokinesis furrow just after formation was complete.

orientations as the spindle formed. In contrast, in most *let-99* embryos, the P_1 nucleus failed to undergo rotation during prophase and the P_1 spindle set up transversely (compare Figs 1D,I, and 2B,F). Thus, in about 40% of *let-99* mutant embryos, the second cleavage spindle orientations were reversed with respect to wild type. The reversed pattern usually led to abnormal cellular arrangements (Figs 1J, 2G).

Alterations in spindle orientations were also seen at third cleavage in *let-99* mutants. In wild-type embryos, the AB daughter cells have transverse spindles (Figs 1E, 2D). In the AB daughters of *let-99* mutant embryos, the spindles were usually oriented with one pole towards the membrane that separates the AB daughters; that is, the spindles were oriented on the same axis as at the previous division (Figs 1J, 2H; 16/17 for ABa, 10/17 for ABp). The ABa and ABp orientations were independent of whether AB divided normally or abnormally at second cleavage. In wild-type embryos, the EMS and P_2 nuclei rotate 90° and 45°, and have longitudinal and dorsal-ventral spindle axes, respectively. In *let-99* mutants, the P_1 daughter cell nuclei often failed to undergo rotation and spindles were positioned transversely (compare Fig. 1E,J); this was most easily scored in the anterior P1 daughter (10/15 transverse). The cell cycles of the P_1 daughters were also perturbed (described later). Analysis of later divisions was prevented by abnormalities in cell position caused by the early alterations.

In *let-99* mutant embryos, many cells exhibit abnormal spindle orientations, but several observations suggest that spindle orientation is not simply random. First, in the AB lineage, one pole of the 'longitudinal' spindles appeared to be orienting to a site on the posterior cortex of AB in a manner similar to that seen in a wild-type P_1 cell during nuclear rotation**.** Spindle orientation was often late in AB, but we observed clear cases of nuclear rotation during prophase in ABa. Second, although spindle orientation in AB was quite variable, ABa spindles oriented on the same axis as the previous spindle in over 90% of embryos and P_1 failed to undergo nuclear rotation in 90% of embryos. In addition, the different spindle orientations seen in 2-cell *let-99* embryos do not appear to be a secondary consequence of the difference in cell size between AB and P_1. We found no correlation between cell size and longitudinal orientation of the spindle in the AB cells or in the few cases where the P_1 spindle was longitudinal (not shown). As a further test for the effects of cell size, we examined *let-99 par-1* double mutant embryos. In *par-1* and *let-99 par-1* double mutant embryos, first cleavage was more equal than in wild type; however, the second cleavage spindle orientations in the double mutant were like those of *let-99* single mutants (Table 5). Thus, overall there appeared to be a trend towards a reversal of spindle orientations in the AB versus the P lineages in *let-99* mutant embryos.

Instability of nuclear and spindle position

Time-lapse videomicroscopy also revealed that, in *let-99* embryos, the nuclear-centrosome complexes rocked back and forth within the cytoplasm of the P_0, P_1 and AB cells. This rocking action was not restricted to the plane of normal rotation and was different from the movements of wild-type P1 nuclei during rotation. Rocking in *let-99* embryos appeared most vigorous during prophase but continued as the metaphase spindle formed. The nuclei in cells at third cleavage appeared to undergo some rocking, but the motion was diminished relative to that in the first two cell cycles. Spindle position in

Table 3. Spindle orientations at second cleavage

Genotype	*n*	% 'Wild type' AB transverse P_1 longitudinal	% AB and P_1 both transverse	% 'Reversal' AB longitudinal P_1 transverse	% AB and P_1 longitudinal
wild type	8	100	0	0	0
let-99(it141)	19	0	53	37	11
let-99(it141) / sDf22	14	14	57	29	0
let-99(s1201)	19	0	58	37	5
let-99(s1201) / sDf22	14	0	29	64	7

In these experiments, mounting conditions were used that avoid flattening the embryos. See methods for details of scoring orientations.

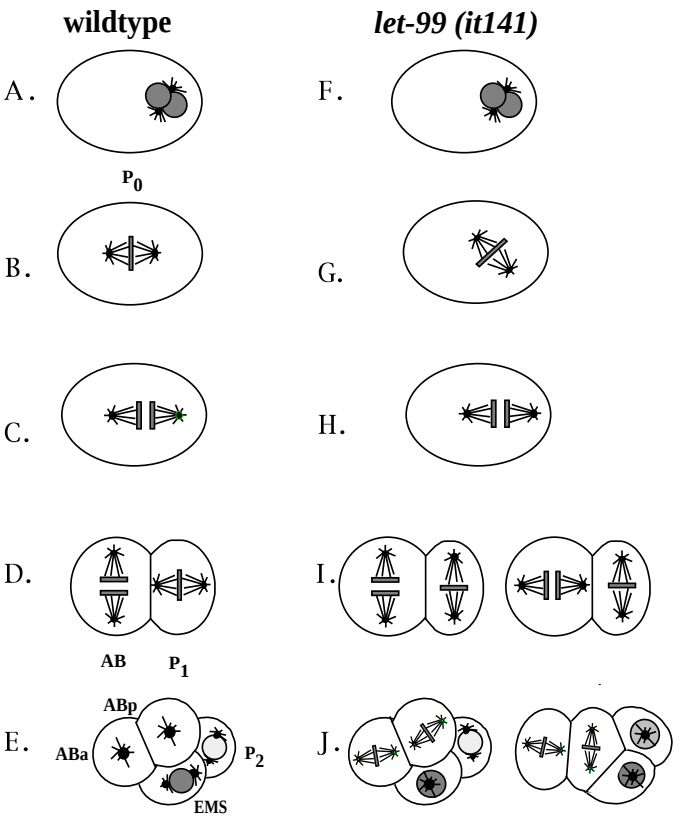

Fig. 1. Schematic summary of cleavage patterns based on time-lapse videomicroscopy. Centrosomes and microtubules are represented by small filled circles and lines, respectively. The lengths of the astral microtubules have been greatly reduced for clarity. Nuclei are shown as circles, shaded light to indicate interphase, dark for prophase; single and double bars represent metaphase and anaphase chromosomes. Posterior is to the right in this and all subsequent figures. First cleavage: position of pronuclear meeting (A,F), initial spindle position (B,G) and final spindle position (C,H). Second cleavage: (D,I) the two major phenotypes of *let-99* embryos are shown. Third cleavage: (E,J) two examples of third cleavage in *let-99* mutants are shown. Other embryos show different combinations of the spindle orientations shown.

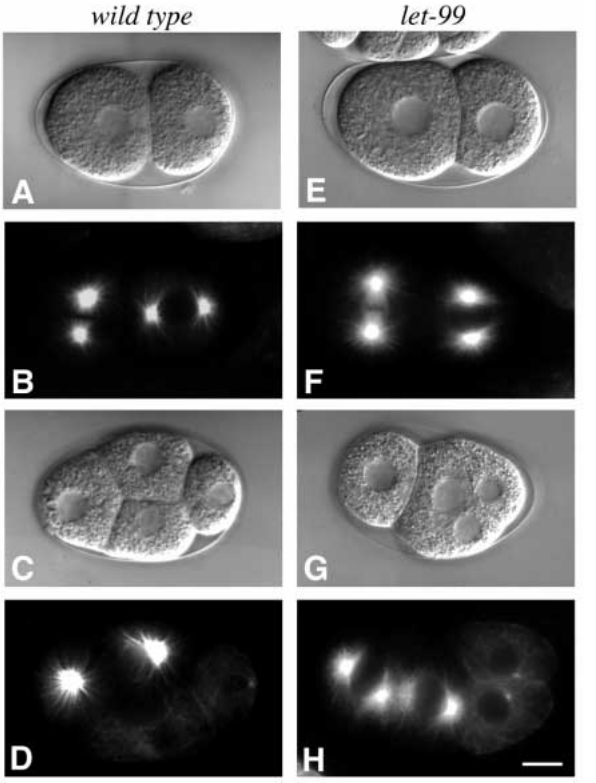

Fig. 2. Wild-type and *let-99* mutant embryos during early cleavage. 2-cell (A,E) and 4-cell (C,G) living embryos viewed with Nomarski optics. Embryos undergoing second cleavage (B,F) and third cleavage (D,H) fixed and stained for anti-tubulin immunofluorescence; the brightest regions are the spindle poles. Scale bar: 10 μm.

let-99 embryos was also easily perturbed by mounting conditions. It has been observed that longitudinal spindle orientations are less frequent in *par-3* mutant embryos that are mounted on agar pads and flattened slightly by the weight of a coverslip (Cheng et al., 1995); slight pressure does not perturb spindle orientations or the development of wild-type embryos. We examined *let-99* embryos mounted on an agar pads and found that the AB spindle was longitudinally oriented much less often than in 'unflattened' *let-99* embryos (Table 5).

Other aspects of asymmetry are normal in *let-99* mutants

The tendency towards reversal of spindle orientation pattern in *let-99* mutants does not appear to reflect a more general defect in embryonic polarity. Second cleavage is asynchronous as in wild type, with AB dividing before P_1 (Fig. 1). We also examined the distribution of P granules as a marker for polarity. In wild-type embryos, germ-line-specific P granules become localized to the posterior pole of the embryo during the first cell cycle, and so are partitioned into the P_1 cell (Fig. 3A). The P granules are then segregated to the P daughter at each of the next three divisions (Fig. 3B; Strome and Wood, 1983). In 1-cell and 2-cell *let-99* mutant embryos (Fig. 3D), the P granules were localized normally to the posterior (98%, n=81; 100% for wild type, n=37). Due to the abnormal cleavage plane in P_1, however, the subsequent distribution of the P granules in older embryos was abnormal (Fig. 3E). As an additional marker of asymmetry, we examined the distribution of the PAR-1 protein, which is required for normal polarity in the early embryo. PAR-1 protein is localized to the posterior periphery of the 1-cell and the P cells (Guo and Kemphues, 1995). In *let-99* 1-cell and 2-cell embryos, the PAR-1 protein was localized normally (97%, n=35; 100% for wild type, n=43).

To determine if the asymmetry in cell fate potential produced by first cleavage is normal in *let-99* embryos, we carried out laser ablation experiments. In wild-type embryos, the AB and P_1 cells give rise to different cell types, a subset of which can be produced autonomously (Priess and Thomson, 1987; Mello et al., 1992). If the AB nucleus is ablated, the partial embryos produced by the 'isolated' P_1 has gut cells, body wall muscle, pharynx, hypodermis and neurons. AB will produce only hypodermis and neurons when P_1 is ablated; production of AB-derived muscle and pharynx requires cell interactions with P_1 descendants. We ablated either the AB or P_1 nucleus in wild-

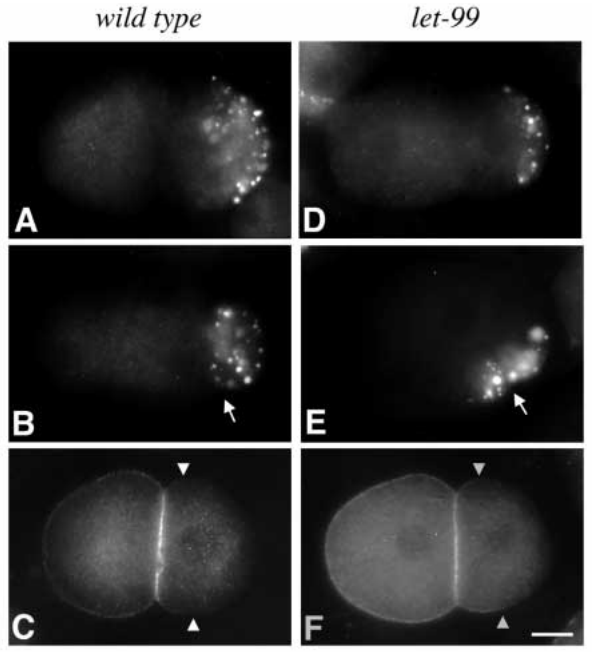

Fig. 3. Immunolocalization of P granules and PAR-3. 2-cell embryos (A,D) in which P granules are localized to the posterior. Wild-type 4-cell embryo (B) in which all P granules are present in the P_2 cell. *let-99* mutant embryo (E) in which P granules are present in both posterior daughters. Arrows point to the boundary between the P_1 daughter cells. 2-cell embryos (C,F) in which PAR-3 protein is all around the periphery of the AB cell, and on the anterior periphery of the P_1 cell. Arrowheads indicate the limits of the PAR-3 protein in the P_1 cells. Scale bar: 10 μm.

type and *let-99* mutant embryos and allowed the partial embryos to develop to term. In most ablated *let-99* embryos, isolated P_1 cells produced muscle, gut and pharyngeal tissue, while isolated AB cells produced only hypodermis and neurons (Table 4). Inappropriate cell types were never produced from AB, indicating that cell fate potentials were not reversed in *let-99* embryos.

From the examination of polarity markers and cell fate specification, we conclude that the *let-99(it141)* mutation affects nuclear positioning and spindle orientation, but not other asymmetries. Embryos from *let-99(s1201)* homozygous and *let-99(it141)/sDf22* hermaphrodites showed similar phenotypes with respect to asymmetries in all cases examined (posterior pronuclear meeting, cell cycle asynchrony, P granule localization). Thus, we believe that the *let-99* gene is required for controlling spindle orientations after polarity establishment.

The P_1 division in many *let-99* embryos retains some asymmetry

let-99 embryos die as masses of differentiated cells, with little or no morphogenesis (not shown). Embryos examined for differentiated tissues had hypodermis and neurons, and many embryos produced body wall muscle (22/26), pharynx (11/26) and gut (17/26). The average number of gut cells in those embryos (20.3±3.9, n=6) was similar to that in wild type (20, Sulston et al., 1983), which was initially surprising. In wild-type embryos, the EMS cell inherits the potential to produce gut cells and pharyngeal tissue, and gut determination also requires induction from the P_2 cell (Laufer et al., 1980; Goldstein, 1993). We expected the failure of P_1 nuclear rotation in *let-99* mutants to cause a symmetric division that produced either two EMS-like cells (which might form 40 gut cells) or two P_2-like cells (yielding 0 gut cells), as is seen in some cell fate determination mutants (Bowerman et al., 1992; Mello et al., 1992). In order to determine if the P_1 division in *let-99* embryos produced hybrid daughters, both producing half of the intestinal cells, we carried out laser ablations. At the 4- to 6-cell stage in control and *let-99* mutant embryos, we ablated the nuclei of all cells except either the posterior or anterior daughter of P_1. The embryos were then allowed to develop until differentiated tissues could be scored. As shown in Table 4, when gut cells were produced by the unablated blastomere in *let-99* embryos, approximately normal numbers of gut cells were present. This suggests that all of the intestinal cells derived from one blastomere, rather than both daughters. In addition, there was a tendency towards the anterior P_1 daughter producing the gut cells, as it does in wild-type ablated controls.

Observations of other markers at the 4-cell stage indicated that, in many *let-99* embryos, the P_1 division was not

Table 4. Production of differentiated cell types from isolated cells

Cell remaining after ablation	Genotype	Body muscle	Pharynx	Gut	No. of gut cells
P1	*wild type*	10/10	10/10	10/10	19.5±2.0
	let-99	10/10	7/10	8/10	20.6±5.8
AB	*wild type*	0/11	0/11	0/11	
	let-99	0/10	0/10	0/11	
Anterior P1 daughter*	*wild type*	3/5	5/5	5/5	20.1±2.3
	let-99	5/8†	5/8†	5/8†	21.4±2.7
Posterior P1 daughter*	*wild type*	4/5	0/5	0/5	
	let-99	8/9	1/9	1/9	18.0

Results are given as number of partial embryos showing differentiated cell type / total number of ablated embryos. The number of gut cells present in partial embryos is given as the mean ± standard deviation.

*In the majority of *let-99* embryos, one of the P_1 daughters moved into a more posterior position, allowing us to ablate either the anterior or posterior cell as in a wild-type embryo. Embryos in which neither of the P_1 daughters was more posterior were not included. Such embryos were less than 10% of the population and produced similar arrays of differentiated cells.

†The embryos that differentiated muscle, gut or pharynx were not always the same embryos. For example, of the five gut$^+$ embryos, only four were also pharynx$^+$ and three were also muscle$^+$.

symmetric. In 4-cell *let-99* embryos, 17.5% of embryos scored had P granules in only one of the P_1 daughters and 42.1% of the embryos appeared to have more P granules in one of the P_1 daughters (*n*=57). In embryos where one P_1 daughter was positioned more posteriorly, that cell was always the one with more P granules. Similarly, there was more PAR-1 protein on the periphery of the posterior P_1 daughter in 80% of 4-cell *let-99* embryos (*n*=10). Furthermore, the anterior P_1 daughter clearly divided before the posterior daughter in 65% of *let-99* embryos (*n*=37), similar to division of EMS before P2 in wild-type embryos. However, the cell cycle lengths of the two cells were usually much more similar than in wild type and, in the remaining *let-99* embryos, appeared synchronous.

Thus, in *let-99* embryos, the absence of nuclear rotation correlated with the mispartitioning of several factors, but the P_1 division retained some asymmetry in greater than 60% of *let-99* embryos. Since rotation during prophase or metaphase occurred rarely in *let-99* embryos (Table 3), these results suggest that the P_1 spindle became more aligned with the axis of polarity later in the cell cycle. A shift in the P_1 spindle position could be due to a late interaction with the normal anterior cortical rotation site, or could result from one spindle pole interacting with the 'posterior determinant' that normally positions the spindle asymmetrically, or both.

Given the remnant of asymmetry in the P_1 division in *let-99* embryos, this mutant cannot be used to ascertain how an exactly transverse, symmetrical division of P1 would affect the fates of its daughters. However, our results do suggest that mixing some posterior cytoplasm, including P granules, with cytoplasm destined for the EMS cell is compatible with production of intestinal and pharyngeal cell types. This is consistent with previous results showing that the presence of P granules does not preclude the development of somatic tissues (Mello et al., 1992; Strome et al., 1995).

The distribution of PAR-3 and PAR-2 is normal in *let-99* mutant embryos

The *par-3* and *par-2* genes are needed for the establishment of polarity in the embryo, and play an important role in the control of spindle orientation. In wild-type embryos, PAR-3 protein is present around the entire periphery of the AB cell, and on the anterior periphery of P_1 (Etemad-Moghadam et al., 1995). In *par-3* mutants, the AB and P_1 spindles are both longitudinally oriented. In contrast, in wild-type embryos, the PAR-2 protein is present on the posterior periphery of P_1, similar to PAR-1 (Boyd et al., 1996). In *par-2* mutants, PAR-3 is around the periphery of both AB and P_1, neither cells' nucleus undergoes rotation, and both spindles have a transverse orientation. Thus, it appears that PAR-3 inhibits nuclear rotation and PAR-2 restricts that activity to the anterior of the embryo so that, in wild type, the AB nucleus does not rotate, but the P_1 nucleus does. In other *par* mutants, a strong correlation exists between the presence of PAR-3 and absence of rotation (Cheng et al., 1995; Etemad-Moghadam et al., 1995).

To determine if the failure of nuclear rotation in the P_1 cells of *let-99* embryos correlated with an abnormal PAR-3 or PAR-2 distribution, we stained *let-99* mutant embryos with antibodies directed against these proteins. One-cell *let-99* embryos showed the same distribution of PAR-3 as wild type (97%, *n*=32 versus 100%, *n*=21 for wild type). The distribution of PAR-3 in the AB and P_1 cells also appeared normal (Fig. 3C,F; 88%, *n*=16 versus 83%, *n*=12 for wild type). The distribution of PAR-2 in 1-cell and 2-cell *let-99* embryos was also normal (98%, *n*=42 versus 100%, *n*=21 for wild type). Mutant embryos did show abnormal distributions of the PAR proteins after second cleavage, in patterns that were consistent with the altered P_1 division plane and P granule inheritance**.** These results show that the reversed pattern of spindle orientations in *let-99* mutants does not correlate with a change in the asymmetry of PAR-3 or PAR-2 and are consistent with the view that *let-99* mutations do not alter overall polarity.

To further explore the relationship between *let-99* and the *par* genes, we constructed double mutants using putative null alleles of *par-3* and *par-2*. Embryos produced by *par-3; let-99* mothers exhibited characteristics of both single mutants. At first cleavage, the spindle often formed at an angle as in *let-99* embryos, but the cleavage was equal as in *par-3* embryos (not shown). Nuclear-centrosome complexes in the double mutant embryos exhibited the rocking phenotype characteristic of *let-99* embryos. Second cleavage in double mutant embryos resembled that in *par-3* embryos: Cleavage was synchronous and both the AB and P_1 spindles oriented longitudinally in most embryos (Table 5). Since the spindle orientations of both *let-99* and *par-3* single mutants are altered when embryos are 'flattened' on agar pads, we also analyzed the double mutants under these conditions. Surprisingly, the second cleavage

Table 5. Spindle orientations in *let-99 par* double mutants

Genotype	Mounting method	*n*	%'Wild type' AB transverse P_1 longitudinal	% AB and P_1 both transverse	% 'Reversal' AB longitudinal P_1 transverse	% AB and P_1 both longitudinal
let-99	unflattened	19	0	53	37	11
par-1	unflattened	12	92	8	0	0
par-1 let-99	unflattened	14	0	43	43	14
par-3	unflattened	13	0	0	0	100
let-99 par-3	unflattened	14	0	7	0	93
par-2	unflattened	17	18	82	0	0
let-99 par-2	unflattened	17	6	18	23	53
let-99	flattened	23	0	91	9	0
par-3	flattened	18	17	11	17	55
let-99 par-3	flattened	17	23	59	18	0

Embryos were either mounted in a way that avoids pressure (unflattened) or under slight pressure (flattened, see methods). The *let-99* single mutant data is taken from Table 3. *n*=number of embryos.

Table 6. Accumulation of capping protein and actin

Antibody	Genotype	*n*	% Asym. P_1	% Asym. AB	% Sym.	% No accum
CP	*wild type*	36	69.4	2.8	11.1	16.7
CP	*let-99*	35	42.9	5.7	31.4	20.0
actin	*wild type*	27	59.3	3.7	14.8	22.2
actin	*let-99*	24	33.3	12.5	25.0	29.2

Embryos were stained with antibodies against either capping protein (CP) or actin, and the accumulation of complexes at the midbody region between AB and P_1 was scored as belonging to one of three classes: asymmetric (asym.) accumulation in either the AB or P_1 cell, symmetric (sym.) accumulation in AB and P_1, and no increased accumulation (no accum.) relative to the rest of the cortex. *n*=number of embryos.

spindle orientations in the double mutants did not resemble those of *par-3* embryos under these conditions; longitudinal spindle orientations were reduced much more in *let-99 par-3* embryos than in *par-3* single mutants (Table 5). In *let-99 par-2* double mutant embryos, cleavages were symmetric and synchronous as in *par-2* embryos, and showed the *let-99* nuclear rocking phenotype. Interestingly, however, many double mutants embryos exhibited longitudinally oriented spindles in both the AB and P_1 cells, a phenotype seen infrequently in *let-99* mutants and never in *par-2* mutants (Table 5). In summary, there is no simple epistatic relationship between *par-3* and *let-99* or *par-2* and *let-99* and the phenotype of the double mutants is best described as additive.

The distribution of actin and actin capping protein is abnormal in *let-99* mutant embryos

One explanation for the reversed spindle orientations in *let-99* mutant embryos is that some factor required for orienting the spindle is mislocalized in these mutants. We showed above that PAR-3 is localized normally in 2-cell *let-99* embryos. Two other proteins that show an asymmetric distribution in the 2-cell embryo and which have been postulated to play a role in spindle orientation are actin capping protein (CP) and actin. CP and actin accumulate in a 'dot' on the anterior cortex of P_1 near or at the midbody, as well as over the entire cortex of both the AB and P_1 cells (Waddle et al., 1994). The CP/actin dot marks the cortical site where microtubules appear to attach during nuclear rotation, but a role for CP in rotation remains to be shown. We stained wild-type and *let-99* mutant embryos with anti-CP and anti-actin antibodies to determine the location of the cortical dot. The observed staining patterns in both wild-type and *let-99* embryos fell into three general classes: asymmetric accumulation, symmetric accumulation and no increased accumulation at the midbody region (Table 6). Embryos were scored as showing asymmetric accumulation when there was a dot or plaque of CP/actin clearly in the P_1 or AB cell. Embryos were scored as having symmetric accumulation when there appeared to be equal amounts of CP/actin on both sides of the membranes between AB and P_1; this usually appeared as a plaque on both sides, rather than as dots protruding into both cells.

Most wild-type embryos in prophase through metaphase showed an asymmetric accumulation of CP or actin in P_1 (Fig. 4A). Many *let-99* mutant embryos also had an asymmetric accumulation of actin or CP on the anterior cortex of P_1 (Fig. 4B, Table 6); of those embryos, when spindle orientation could be determined, it was always transverse (*n*=12). Relative to wild type, an increased proportion of *let-99* embryos showed asymmetric AB accumulation (Fig. 4D), symmetric accumulation or no accumulation (Table 6). Of those *let-99* embryos in which AB was dividing longitudinally (*n*=8/28), one embryo had AB asymmetric accumulation, one had symmetric, two had P_1 asymmetric, and four showed no accumulation.

Thus, we conclude that, although the distribution of CP was somewhat abnormal in *let-99* mutant embryos, the assembly of CP at the cortical rotation site was not simply reversed, and there was no clear correlation between the accumulation of CP/actin complexes and nuclear rotation in either AB or P_1. If CP/actin accumulation is involved in rotation, then in *let-99* mutant embryos it appears that the P_1 CP/actin complex is either inactive, or the microtubules are otherwise prevented from a making a productive interaction with the rotation site in P_1. In addition, since the symmetric accumulations in both wild-type and *let-99* embryos did not appear as dots protruding into both cells, this class may actually reflect the instability of CP/complexes under the fixation conditions used (see Methods). In this case, the increase in frequency of *let-99* embryos with symmetric or no accumulation of CP/actin could indicate that CP/actin complexes are less stable in this mutant. Otherwise, there was no difference in the size of the plaques and dots seen in *let-99* embryos compared to wild type.

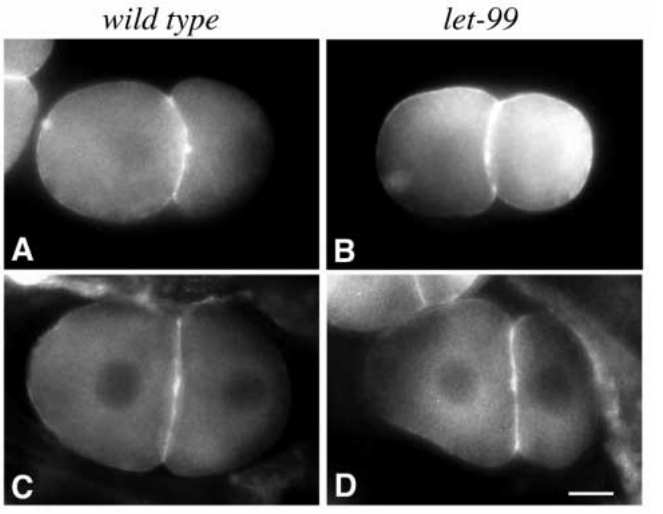

Fig. 4. Actin accumulation at the cortical rotation site in 2-cell embryos. Asymmetric accumulation protruding into the P_1 cell of a wild-type embryo (A) and a *let-99* mutant embryo (B). Symmetric accumulation in a wild-type embryo (C). Asymmetric accumulation in the AB cell of a *let-99* embryo (D). All embryos were in late prophase. Scale bar: 10 μm.

DISCUSSION

Maternal effect lethal mutations in the *let-99* gene of *C. elegans* have dramatic effects on nuclear position and spindle orientation during early cleavage. Spindle orientations in *let-99* mutants are abnormal and, in some cases, are reversed with respect to the wild-type pattern, even though several other aspects of embryonic polarity are normal. The orientation defects lead to abnormal cellular arrangements and disrupt cytoplasmic partitioning, both of which probably contribute to the inviability of the embryos. We propose that the *let-99* gene functions to control spindle orientation, but not to establish other asymmetries. These characteristics indicate that *let-99* is different from the previously characterized *par* genes and *let-99* mutations are among the first mutations that specifically affect spindle orientation to be described in an animal system. The *C. elegans gpb-1* gene, which encodes a G protein β subunit, is required for normal spindle orientation in the embryo, as well as for other processes. The cleavage pattern defects of *gpb-1* -depleted embryos (flattened on agar) bear some similarity to *let-99* mutant embryos (on agar) and P granule localization is initially normal (Zwaal et al., 1996). The similarity in phenotypes raises the possibility that *gpb-1* and *let-99* function in a common mechanism to control spindle orientation, but a more detailed comparison of the mutant phenotypes is needed.

The role of the *let-99* gene

We speculate that the role of *let-99* is to mediate or regulate connections between microtubules and the cortical cytoskeleton. In the P_0, P_1 and AB cells of *let-99* mutants, the nuclear-centrosome complex exhibits a rocking motion. This phenotype and the altered spindle orientations could each reflect abnormal interactions between astral microtubules and the cortex of the cell. For example, LET-99 protein could function as a structural component of the cortical cytoskeleton or as part of the linkage between microtubules and the cortex, or LET-99 could regulate such structural components. The observation that ectopic rotations occur in *let-99* embryos argues against a role for LET-99 in the function of the cortical rotation site per se, but LET-99 could modify its location or activity.

The *par-3* gene has been proposed to play a role in stabilizing spindle position in the early embryo (Cheng et al., 1995; Etemad-Moghadam et al., 1995). PAR-3 acts to inhibit rotation in the AB cell in wild-type embryos. In both *let-99* and *par-3* mutants, the AB spindle can be longitudinal, but flattening embryos reduces the frequency of longitudinal spindle orientations. Unlike *let-99* mutants, however, *par-3* mutants usually have nuclear rotation in P_1 and the nuclear-centrosome complexes do not rock. Finally, there is no clear epistasis of the *let-99* and *par-3* mutations. We conclude that *par-3* and *let-99* perform similar but not identical roles in stabilization and that they do not appear to function in a simple linear pathway for synthesis or function. However, our observations do not rule out the possibility that LET-99 is downstream of PAR-3 in a localization pathway.

We suggest that *let-99* carries out its function in all cells of the early embryo. Why then, does the loss of *let-99* activity have different consequences in the anterior versus the posterior cells? The model that we favor is that the asymmetric distributions of PAR-3 and PAR-2 (and/or other factors) in the AB and P_1 cells lead to different consequences upon removal of *let-99(+)* function. The double mutant results are consistent with this model. In both the *let-99 par-3* and *let-99 par-2* double mutant embryos, the AB and P_1 cells behave more similarly to each other than in *let-99* single mutants. This could be explained in part by the observation that, in *par-2* mutants, PAR-3 is symmetrically distributed at the 2-cell stage and, in *par-3* mutants, PAR-2 is symmetrically distributed (Etemad-Moghadam et al., 1995; Boyd et al., 1996). The unique phenotype of each of the double mutants is presumably because the absences of PAR-3 and PAR-2 have different effects on the distribution of other factors that influence spindle orientation.

Some evidence argues against alternative models for the *let-99* phenotype. For example, one explanation for the *let-99* second cleavage phenotype is that spindle orientation is really variable but, because the P_1 cell is much smaller, or because it divides after AB, the spindle orients transversely. This model is inconsistent with the lack of correlation between cell size and spindle orientation in *let-99* and the data from *let-99 par-1* embryos. Another alternative is that *let-99(+)* normally localizes a factor required to inhibit spindle orientation to the anterior pole (or vice versa). In *let-99* embryos, this factor is mislocalized to the posterior pole in some embryos, producing the reversal phenotype, or is uniformly distributed in some embryos, producing transverse spindles in both AB and P_1. (The *let-99* alleles act as loss-of-function mutations, but mislocalization could occur through the action of cytoplasmic streaming or other asymmetries.) However, PAR-3 and PAR-2 were distributed normally in early *let-99* embryos. In addition, the accumulation of CP and actin on the cortex, which correlates with nuclear rotation in wild-type embryos, was not reversed in *let-99* embryos. The strongest argument against the mislocalization model is that it does not explain the observation that nuclear-centrosome rocking occurs in both the AB and P_1 cells of *let-99* embryos.

Specification of spindle orientation in wild-type embryos

How are the wild-type patterns of spindle orientation controlled? The simplest model would be that factors required for rotation are localized exclusively to the P_1 cell. Conversely, factors that inhibit rotation, like PAR-3, could be localized to the AB lineage. An alternative hypothesis, alluded to above, is that each cell contains multiple factors that influence spindle orientation. We speculate that a specific combination of factors (and/or their distribution) in P_1 facilitates interactions between microtubules and the cortex that allow for efficient nuclear rotation during prophase and then stabilize the spindle in the longitudinal position. A different combination of factors in AB may mediate very stable interactions which prevent nuclear rotation. This combinatorial model is consistent not only with our observations of *let-99*, but also with the fact that PAR-3 is present all around the periphery of EMS, yet that cell shows nuclear rotation (Etemad-Moghadam et al., 1995). The molecular characterization of *let-99* and the immunolocalization of the LET-99 protein, as well as the study of other spindle orientation genes identified in our screen, will be important for testing these ideas.

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