

C. elegans PAR Proteins Function by Mobilizing and Stabilizing Asymmetrically Localized Protein Complexes

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Summary

Background: The PAR proteins are part of an ancient and widely conserved machinery for polarizing cells during animal development. Here we use a combination of genetics and live imaging methods in the model organism *Caenorhabditis elegans* to dissect the cellular mechanisms by which PAR proteins polarize cells.

Results: We demonstrate two distinct mechanisms by which PAR proteins polarize the *C. elegans* zygote. First, we show that several components of the PAR pathway function in intracellular motility, producing a polarized movement of the cell cortex. We present evidence that this cortical motility may drive the movement of cellular components that must become asymmetrically distributed, including both germline-specific ribonucleoprotein complexes and cortical domains containing the PAR proteins themselves. Second, PAR-1 functions to refine the asymmetric localization of germline ribonucleoprotein complexes by selectively stabilizing only those complexes that reach the PAR-1-enriched posterior cell cortex during the period of cortical motility.

Conclusions: These results identify two cellular mechanisms by which the PAR proteins polarize the *C. elegans* zygote, and they suggest mechanisms by which PAR proteins may polarize cells in diverse animal systems.

Introduction

Asymmetric division plays an important role in generating cell diversity during animal development. In the past decade, a number of critical developmental factors that are partitioned in asymmetric divisions have been identified in *Drosophila*, *C. elegans*, and mammals [1–4]. Despite this progress, we know very little about how the asymmetric distributions of these gene products are accomplished in any of these systems.

The potential for combining genetics with live imaging makes the early *C. elegans* embryo an ideal model for addressing the mechanisms of asymmetric division. Before the first cell division in *C. elegans*, P granule ribo-

nucleoprotein complexes, several PAR proteins, and other proteins and mRNAs become asymmetrically localized to one end of the zygote or the other [4]. Later, the mitotic spindle moves to an asymmetric position, and cytokinesis occurs [5]. The result is unequally sized daughter cells containing distinct molecular components [6]. All of these events can be seen in living embryos by either differential interference contrast (DIC) microscopy or fluorescence microscopy of specific fluorophore-labeled proteins.

Near the time that asymmetries first appear, the zygote can be seen to undergo a dramatic reorganization (Figure 1A). The actin cortex moves anteriorly, and yolk granules and vesicles visible by DIC microscopy move in concert with the actin cortex, away from the posteriorly localized sperm asters [7–9]. Central cytoplasm moves posteriorly, most likely driven by the displacement of actin cortex anteriorly because both the cortical and central cytoplasmic movements depend on a cortically enriched myosin II, *nmy-2* [10]. The zygote during the period of flow resembles a crawling cell in that the actin cortex and the central cytoplasm are moving in opposite directions relative to each other [11–13]. When cells crawl, cortical and central cytoplasmic flow result in the circulation of many intracellular components, and a similar circulation of contents occurs in the *C. elegans* zygote during the period of flow [7, 8].

The PAR proteins are part of a conserved machinery for polarizing cells in *C. elegans*, *Drosophila*, *Xenopus*, and mammals [4, 14–16]. Certain of the PAR proteins are localized asymmetrically in *C. elegans* before the first cell division. PAR-3, which has three PDZ domains, interacts with PAR-6, which also has a PDZ domain, and PKC-3, an atypical protein kinase C. These proteins localize to the anterior cortex and form a putative intracellular signaling complex with CDC-42. PAR-2, which has ring finger and ATP binding domains, and PAR-1, which is a serine threonine kinase, localize to the posterior cell cortex. PAR-4, a serine threonine kinase, and PAR-5, a 14-3-3 protein, localize to the entire cell cortex. Loss of function of any of these proteins in *C. elegans* embryos results in a failure to establish either some or all embryonic asymmetries (Figure 1B; [15]).

Little is known about the cellular and molecular mechanisms by which the PAR proteins polarize cells in any organism. Interactions between members of the PAR-3/PAR-6/PKC-3/CDC-42 complex have been identified in several systems [16], and PAR-1 can interact with both NMY-2 and PAR-5 [17, 18]. In *C. elegans*, no biochemical targets outside of these complexes have yet been identified. Targets of PAR-1's kinase activity have been identified in *Drosophila*; *Drosophila* PAR-1 can phosphorylate 14-3-3 binding sites on proteins [18] as well as the posterior determinant Oskar, resulting in the protection of Oskar from degradation in the posterior of the embryo [19].

There have been few studies of the *in vivo* localization of asymmetrically distributed proteins in animal developmental systems. The optical clarity of the *C. elegans*

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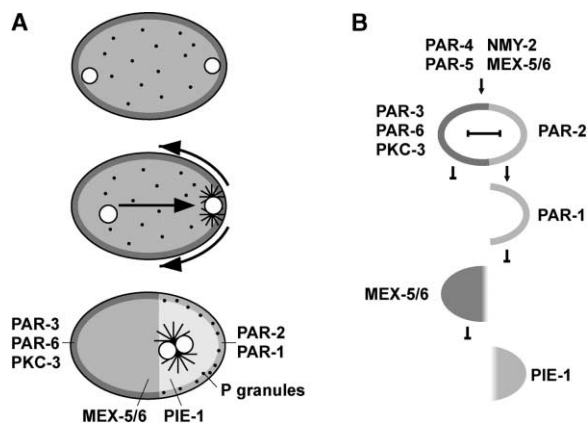


Figure 1. Establishment of Asymmetry in the *C. elegans* Embryo
(A) Schematic diagram of steps in early development. (Top) Fertilized embryo with egg pronucleus (left) and sperm pronucleus (right). (Middle) Period of cortical and central cytoplasmic flow. (Bottom) Some of the asymmetric proteins and protein complexes are labeled at the stage of pronuclear meeting. Anterior is to the left in all figures. (B) Genetic pathway of proteins involved in generating asymmetry (after [4, 22]).

embryo makes it ideal for such studies. In *C. elegans*, P granule movements have been examined during the early stages of wild-type embryos [20]. Because P granules have a particulate distribution, time lapse imaging of fluorescently labeled P granules could distinguish whether P granules redistribute by moving or by being locally eliminated. At the one-cell stage, P granules redistribute primarily by moving, although near the end of the period of movement, mislocalized P granules are eliminated, presumably by either degradation or disassembly of P granule components.

Two other studies used live fluorescence imaging to examine the roles of PAR proteins in generating asymmetries in *C. elegans* [21, 22]. These studies examined localization patterns of proteins that initially had uniform cytoplasmic distributions and subsequently became asymmetrically localized. Although the time course of reorganization could be tracked, it was not determined whether each change in localization was a result of protein movement, localized degradation, and/or localized synthesis. Movements of proteins and the mobile cellular components to which certain proteins bind can be made visible either by examination of particulate assemblies of proteins over time or, for proteins with distributions that appear to be nonparticulate, by analysis of fluorescence recovery after photobleaching (FRAP) of fluorescently labeled proteins [23–25].

Here we take advantage of *C. elegans* genetics and the microscopic clarity of the *C. elegans* zygote to address whether the PAR proteins polarize cells by functioning in intracellular motility. We have examined the movements of cytoplasmic yolk granules and vesicles, P granules, and PAR protein domains and conducted FRAP experiments on GFP-conjugated PAR proteins, both in living embryos. We report that PAR-2, PAR-3, PAR-6, PAR-4, and MEX-5/6 are required for polarized intracellular motility, and the downstream-acting proteins PAR-1 and PIE-1 are not. We present evidence that the polarized cytoplasmic flow directed by these

proteins moves P granules and that cortical flow produces the distinct cortical domains with which specific PAR proteins dynamically associate. P granule localization is refined by a separate mechanism, in which PAR-1 stabilizes only those P granules that have reached the posterior cortex. Our results demonstrate that the PAR proteins polarize the *C. elegans* zygote by two distinct mechanisms and suggest a model by which PAR proteins might function to polarize other cells.

Results

par-2, *par-3*, *par-4*, *par-6*, and *mex-5/6* Function in Intracellular Motility

To measure cortical and central cytoplasmic motility in embryos, we generated kymographs from time lapse DIC videomicroscopy recordings. Kymographs facilitated the quantification of rates and durations of movement of yolk granules and vesicles that can be resolved by DIC microscopy. Yolk granules and vesicles are useful markers for detecting movements of the actin cortex because they have been found to move in concert with the actin cortex [8]. Both cortical and central cytoplasmic regions were analyzed together in each kymograph so that movements of the cortex and central cytoplasm in opposite directions could be identified (Figure 2A). We measured central cytoplasm moving in wild-type embryos at a rate of $4.0 \pm 0.5 \mu\text{m}/\text{min}$ (Table S1 in the Supplemental Data available with this article online), consistent with an earlier estimate based on tracing the movements of individual granules in an embryo ($4.4 \pm 0.8 \mu\text{m}/\text{min}$) [7]. The cortex was observed to move at a similar rate in the posterior half of the embryo, as shown previously [7], and the rate of cortical and central cytoplasmic movement did not change markedly through the time during which these movements occurred (Figure 2A). We found that these movements occurred for a duration of $7.0 \pm 1.8 \text{ min}$ in wild-type embryos (Table S1), resulting in central-cytoplasmic yolk granules or vesicles travelling an average distance of $28.0 \pm 8.0 \mu\text{m}$, a little more than half of the length of the approximately 50- μm -long embryo.

To test whether *par* genes are required for generating this polarized intracellular motility, we generated kymographs from DIC recordings of *par* mutant embryos. Previous experiments concluded that only *par-3* was essential for these movements, but these experiments were conducted on only a subset of the *par* genes, and before information had been obtained to indicate which alleles were likely to be null alleles [26, 10]. We used null alleles where possible, and we used multiple alleles to further test certain conclusions (see Experimental Procedures). We found that little or no polarized intracellular motility occurs in *par-2*, *par-3*, *par-4*, and *par-6* mutant embryos (Figure 2). Among these, *par-2* mutants produce the most cytoplasmic flow, with the cytoplasm generally moving at a speed similar to that observed in wild-type embryos but for about one-fifth the duration (Figure 2, Table S1). The *pie-1* gene, which acts downstream of the *par* genes to generate a subset of embryonic asymmetries [27], is not required for cortical and central cytoplasmic motility (Figure 2). Our results sug-

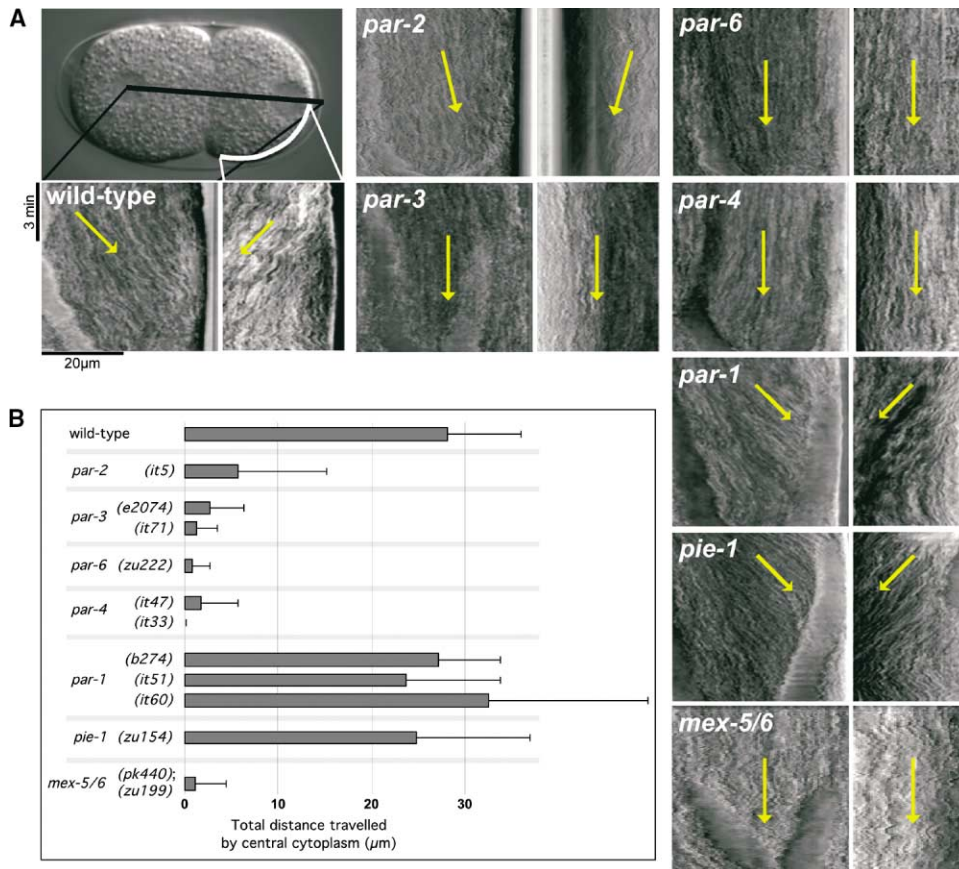


Figure 2. PAR-2, -3, -4, and -6 and MEX-5/6 are Required to Generate Cytoplasmic Flow

(A) Kymographs of flow of yolk granules and vesicles in wild-type and in representatives of seven different mutant backgrounds. In the kymographs, a linear region of the embryo is displayed along the X axis (distance along a chosen line), and linear regions from successive frames are laid down sequentially along the Y axis (time). Kymographs were made from a straight line in the axis of central cytoplasmic flow (black line, left side of each kymograph) and from a curved line along the cortex (white line, right side of each kymograph). Movement along a line results in a diagonal trace, with the slope (distance/time) indicating the rate of movement. A lack of movement results in a vertical trace. A yellow arrow over each trace gives an interpretation of the rate of cortical and central cytoplasmic flow. Time and distance scales are the same for all images, and anterior is on the left in all images.

(B) The product of the average rate and duration of flow in 7–12 embryos for each mutant background was calculated to produce the average total distance travelled by central cytoplasmic yolk granules and vesicles. Mutant alleles of each gene are in parentheses. Error bars represent one standard deviation. See Table S1 for details.

gest that a large part of the known *par* pathway upstream of *pie-1* is required for polarized intracellular motility.

MEX-5 and MEX-6, two similar and partially redundant CCHZ zinc finger proteins, act downstream of the PAR proteins to generate certain aspects of cell polarity [28]. Recent results suggest that these proteins also act at an earlier step, where they establish embryonic polarity together with the PAR proteins [22]. *mex-5/6* double mutant embryos have a symmetric distribution of P granules [28], and we found that the distribution is often symmetric even as early as at the late one-cell and two-cell stages (data not shown). Furthermore, we found that *mex-5(zu199);mex-6(pk44)* double mutant embryos sometimes have Par (partitioning) phenotypes, including equal first cleavage (7/10 embryos), failure of centrosome/nuclear rotation in the P₁ cell (8/11 embryos), and synchronous second division (8/11 embryos). These results suggest that the MEX-5/6 proteins might collaborate with the PAR proteins in generating intracellular

motility. We therefore examined cortical and central cytoplasmic motility in *mex-5/6* double mutant embryos. We found that, as for the *par* genes discussed above, loss of *mex-5/6* also results in little or no cortical and central cytoplasmic motility (Figure 2; Table S1). Consistent with this, the PAR-2 domain is generally small at the stage of pronuclear meeting, when cortical and central cytoplasmic flow would normally end in *mex-5/6* mutants [22]. Although *mex-5/6* mutants have both Mex (muscle excess) [28] and Par phenotypes, for convenience we refer to MEX-5/6 as PAR proteins in this paper.

Germline Ribonucleoprotein Complexes Move Posteriorly in the Cytoplasmic Flow

Because movement of cytoplasm on the scale of a *C. elegans* embryo is predicted to be dominated by viscous forces [29–31], cortical and central cytoplasmic flow should result in the circulation of most cellular compo-

nents. However, the average distance traveled by a visible central cytoplasmic component during the period of flow is only about half of the length of the embryo (see above). We hypothesized that this limited amount of polarized movement of cytoplasm might result in the segregation of central cytoplasmic protein complexes to the posterior half of the embryo and of cortical protein complexes to the anterior half of the embryo rather than in the circulation of all components throughout the embryo. Developmentally critical complexes of proteins are present in the central cytoplasm or the cortex; near the time that flow begins, P granules become enriched in the central cytoplasm ([20] and Movie 2 in the Supplemental Data), and certain PAR proteins are enriched in the cell cortex [6]. Therefore, to test this hypothesis, we have studied the movements of P granules and PAR proteins in wild-type and mutant embryos.

If P granules are moving passively in a viscous cytoplasmic flow, P granules and neighboring cytoplasmic yolk granules and vesicles should move at precisely the same rate and trajectory. PGL-1 is a component of P granules [32]. We generated an integrated GFP::PGL-1 transgenic strain (see Experimental Procedures) and imaged embryos from this strain by simultaneous fluorescence and DIC microscopy. Overlays of fluorescence and DIC recordings revealed that P granules and neighboring yolk granules and vesicles move with the same rate and trajectory during cytoplasmic flow, each P granule remaining in a constant neighborhood of yolk granules and vesicles as it moved toward the posterior (31/31 P granules, ten embryos; Figure 3A).

To test further whether P granules move passively in cytoplasmic flow, we used RNA interference to reduce the function of a cortically enriched myosin II motor, *nmy-2*, that is required for cortical and central cytoplasmic flow [10]. Others have shown by immunostaining fixed embryos that P granules do not become asymmetrically distributed during the period of cytoplasmic flow in the absence of NMY-2 [10], but whether the P granules remained symmetric because they failed to move posteriorly, because they circulated throughout the embryo, or because they moved posteriorly but others reformed in the anterior could not be determined in the absence of live imaging. We recorded fluorescence and DIC images in living GFP::PGL-1 *nmy-2(RNAi)* embryos. Our recordings demonstrated that P granules failed to move posteriorly, indicating that *nmy-2* is required for the polarized movement of P granules (Figure 3B). These results suggest that the myosin-dependent cortical and central-cytoplasmic flow delivers P granules to the posterior of the embryo and that P granules fail to adopt an asymmetric distribution in many of the *par* mutants at least in part because cortical and central cytoplasmic flow fails (and possibly also because PAR-1 fails to localize normally in the absence of cytoplasmic flow; see below).

PAR-2 and PAR-6 Dynamically Associate with Distinct Cortical Domains Generated during Cortical Flow

During the period of flow, cortical actin foci move to the anterior of the embryo, new actin foci subsequently form

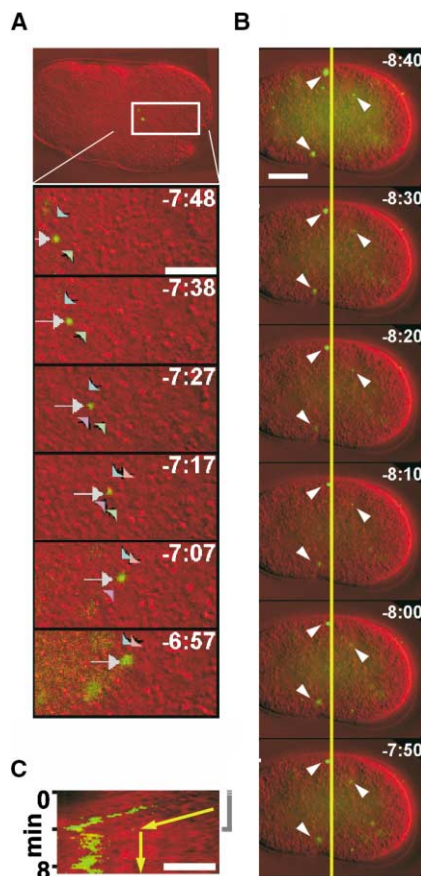


Figure 3. Coordinated Movement of P Granules and Neighboring Yolk Granules and Vesicles

(A) Overlay of fluorescence (green) and DIC (red) images show a P granule moving posteriorly in concert with neighboring yolk granules and vesicles. Yolk granules or vesicles that can be seen in this plane in at least three successive frames are marked with colored arrowheads; the P granule is marked with a gray arrow. Time is shown in minutes and seconds before pronuclear meeting. The scale bar represents 5 μm . Anterior is to the left.

(B) P granules do not move posteriorly in an *nmy-2(RNAi)* embryo. Three P granules are marked with arrowheads; the yellow line is a stationary reference. Time is shown in minutes and seconds before pronuclear meeting. The scale bar represents 10 μm . Anterior is to the left.

(C) Cortical P granules stop moving as cortical flow stops. A gray bracket at the right labels the last 4 min of cortical flow in this kymograph. Cortical yolk granules and vesicles moved with the yellow arrows in the recording (diagonal, moving; vertical, stopped). A P granule (green) can be seen moving in concert with flow, anteriorly along the cortex, and stopping at the same time as flow stopped. The scale bar represents 5 μm . Anterior is to the left.

in the posterior cortex [33, 8], and yolk granules and vesicles enter the posterior cortex from the central cytoplasm [7]. These findings have suggested that the zygote's actin cortex recedes to the anterior half of the embryo and that new actin microfilaments polymerize in the posterior cortex, much as occurs when new microfilaments polymerize at the leading edge of a crawling cell [12]. For convenience, we refer to the posterior cortical region generated during this time as "new cortex." We refer to the anterior region, to which the existing

cortical actin foci, yolk granules, and vesicles associate, as “old cortex.”

PAR-2 is known to associate with posterior cortex near the time that cortical and central cytoplasmic flow occurs [34, 22]. It is possible that PAR-2 directly or indirectly associates with the new actin cortex in the posterior of the embryo and that the asymmetric position and extent of the PAR-2 domain is determined by the position and extent of new cortex generated during flow. If this is the case, it could suggest a mechanism by which cortical and cytoplasmic flow may generate specific cortical domains that polarize the zygote. We tested this by imaging GFP::PAR-2 [35] and cortical and central cytoplasmic flow simultaneously in living embryos. GFP::PAR-2 first becomes enriched in the posterior cell cortex in a small cap, starting 3.5 ± 2.0 min ($n = 8$ embryos) after cortical flow begins (Figure 4A). We found that the extent of this cap coincides with the extent of new cortex, as determined from the separation of yolk granules and vesicles at the posterior pole in time lapse recordings and in kymograph analyses (Figure 4B and Movie 1). As the domain of new cortex expands, the extent of the PAR-2 domain expands with it, and the leading edge of new cortex and PAR-2 coincides with the pseudocleavage furrow. These results suggest that PAR-2 associates directly or indirectly with the new actin cortex formed during cortical and central cytoplasmic flow. Together with the findings that forming a full-sized PAR-2 domain depends on *nmy-2* [10, 22] and that producing further flow by mislocalizing the mitotic apparatus during an abnormal mitosis in *mbk-2* (minibrain kinase 2) RNAi embryos can extend the PAR-2 domain [36, 37], these results raise the possibility that the extent of cortical flow may determine the extent of the PAR-2 posterior domain.

PAR-6 may associate directly or indirectly with the old actin cortex that regresses anteriorly during flow; the posterior margin of the cortical PAR-6 domain also aligns with the pseudocleavage furrow and with the anterior edge of the PAR-2 domain in embryos fixed during or after the period of flow ([38] and data not shown) and in dual-fluorescence/DIC recordings of living GFP::PAR-6 embryos (E. McCarthy and B.G., unpublished data).

PAR-2 might stably associate with new cortex as the cortex moves, or it might dynamically associate with a more stable component of the new cortex. To distinguish between these possibilities, we used a laser to photobleach a selected region of GFP::PAR-2-containing cortex in embryos undergoing cortical and central cytoplasmic flow (Figure 5). If PAR-2 stably associates with new cortex as the cortex moves, then the spot of photobleached GFP::PAR-2 would be expected to remain coherent and move with cortical flow. If, instead, PAR-2 dynamically associates with a more stable component of the new cortex, then photobleached GFP::PAR-2 would be expected to exchange with fluorescent GFP::PAR-2, and fluorescence would fill the photobleached spot. Integrated intensity measurements of a section of cortex are presented in Figure 5B. Photobleached regions of the cell cortex recovered quickly; they equilibrated with nonphotobleached regions of the cell cortex with a half-life of approximately 15 s. Within

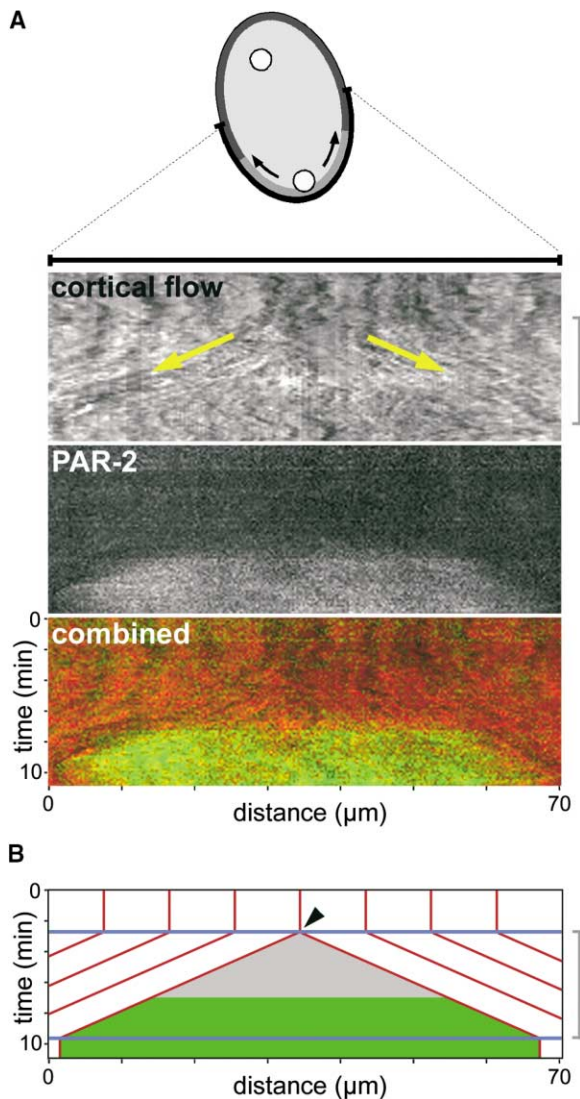


Figure 4. PAR-2 Associates with New Cortex Generated during Flow

(A) Kymographs from a recording of a GFP::PAR-2 embryo. Top to bottom: diagram of embryo from which kymographs were made, kymograph of cortical flow, kymograph of GFP-labeled PAR-2 in the same region, and combined image. A curved black line in the diagram marks the cortical region from which the kymograph was made. This curve was linearized to make the kymograph. Time and distance scales are the same for all images, and distance is a perimeter distance along the cortical region. One frame was recorded every 5 s. See also Movie 1.

(B) Diagram of results. Duration of flow, observed in the top image, is marked with a gray bracket on the right side. Blue horizontal lines mark the beginning and end of the period of flow. Red lines represent diagrammatically the flow trajectories traced from the cortical-flow image. The region from which the cortex began to spread is marked with an arrowhead. This region coincides with the position of the sperm pronucleus in the recordings, as expected, because cortical flow spreads from the position of the sperm pronucleus [9]. Red diagonal lines descending from the arrowhead represent the leading edge of new cortex. The horizontal distance between these lines at any time is the extent of new cortex (area marked in gray plus the area marked in green in the diagram). The green area is where GFP::PAR-2 appeared in this embryo: in the area of new cortex, starting approximately 4 min after cortical flow began.

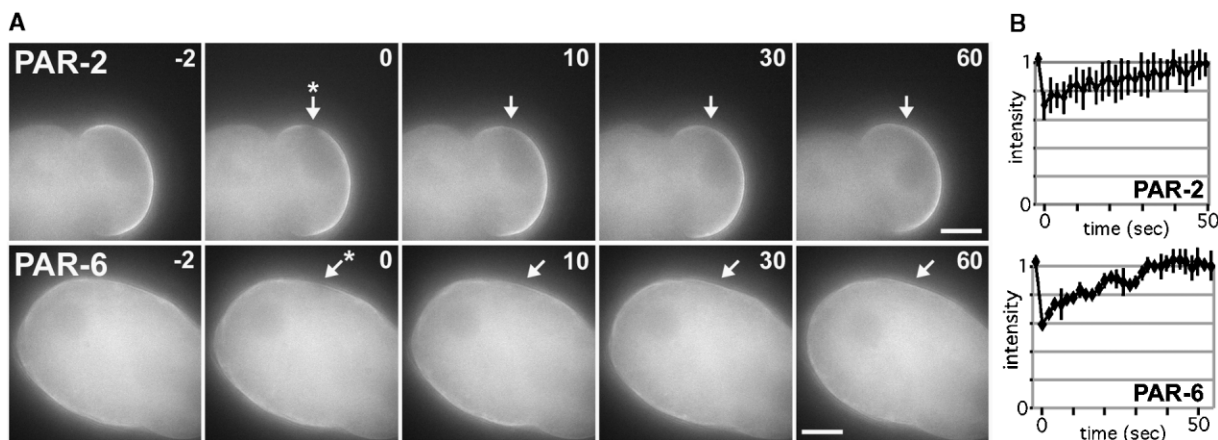


Figure 5. PAR-2 and PAR-6 Dynamically Associate with the Cell Cortex

(A) Photobleached GFP::PAR-2 (top) and GFP::PAR-6 (bottom) embryos. The first frame is a fluorescence image of an embryo before photobleaching, then immediately after photobleaching, then during recovery. An asterisk marks a photobleached spot immediately after photobleaching, and an arrow marks this region over time. Time is shown in seconds before or after the first frame was recorded immediately after photobleaching. Duration of laser treatment (95 ms) was the minimum required to bleach the cortical fluorophore to below cytoplasmic levels; this duration was chosen so that the chance of damage to fluorophore-labeled proteins or neighboring cellular structures would be minimized [23]. After photobleaching and monitoring of fluorescence recovery, embryos survived and developed normally. The scale bars represent 10 μm .

(B) Measurements of maximal fluorescence intensity integrated over an 8-pixel (approximately 0.5 μm)-wide region of cortex recorded every 2 s for GFP::PAR-2 (top; means \pm standard deviations of four embryos) and GFP::PAR-6 (bottom; means \pm standard deviations of two embryos). The y axis is the ratio of intensity in the photobleached region of cortex over the unphotobleached region of cortex from the same embryo. The recovery of intensity to a ratio of 1 suggests that neither protein has a significant immobile fraction at the cell cortex. Because cortex moves at up to about 4 $\mu\text{m}/\text{min}$ (Figure 2A and [7]), we repeated measurements for moving areas and found similar results (data not shown), suggesting that recovery is not caused by the movement of cortex into the region being analyzed.

about 1 min, diffraction-limited photobleached spots completely equilibrated with nonphotobleached areas of cortex, indicating that all or nearly all PAR-2 molecules at the cortex are dynamic. Because recovery was essentially complete, we conclude that there is not a significant immobile fraction of PAR-2 at the cell cortex. These results suggest that PAR-2 protein rapidly exchanges, either between the cell cortex and the cytoplasm or laterally along the cell cortex, while cortical flow occurs. Photobleaching GFP::PAR-6 during the period of flow produced similar results (Figure 5). We conclude that PAR-2 and PAR-6 dynamically associate with unknown components of the new and old cortex, respectively, during cortical flow.

PAR-1 Is Not Required for Cortical and Central Cytoplasmic Flow

Like other *par* mutants, *par-1* mutants have mislocalized P granules [39]. Because cortical and central cytoplasmic flow depended on other *par* genes, we expected that cortical and central cytoplasmic flow would also depend on *par-1*. We generated kymographs from DIC recordings of *par-1(b274)* mutant embryos and were surprised to find that cortical and central cytoplasmic flow appeared normal, with both the duration and the rate of cytoplasmic flow being similar to those in wild-type embryos (Figure 2). To ensure that these results were not an artifact of partial loss of *par-1* function, we similarly examined *par-1(it60)*, a *par-1* allele that lacks detectable PAR-1 protein on Western blots, and *par-1(it51)*, an allele that is predicted to lack kinase activity because an arginine is replaced at a conserved position (K. Kemphues, personal communication, and [40]). Both

of these alleles also had cortical and central cytoplasmic flow that was indistinguishable from that of the wild-type in both duration and rate (Figure 2 and Table S1). We conclude that PAR-1 is not required for cortical and central cytoplasmic flow.

P Granules Do Not Become Trapped When They Reach the PAR-1 Cortical Domain

If P granules do not become asymmetrically distributed in the absence of PAR-1, and yet the flow that appears to carry P granules is normal, how then does PAR-1 function to asymmetrically localize P granules? At the end of the period of cortical and central cytoplasmic flow, PAR-1 and P granules occupy a similar domain at the posterior cell cortex [40]. PAR-1 might function as part of a trap for P granules that reach the posterior cortex and thus prevent the P granules from leaving the PAR-1 cortical domain as flow continues to occur. If this were the case, one would expect P granules to either slow down or stop at the posterior cortex before the time that cortical flow ended. We examined P granules and cortical flow simultaneously in living embryos and found that cortical P granules moved in concert with neighboring yolk granules and vesicles and that P granules do not slow down or stop moving until cortical flow stops (14/14 P granules from eight embryos; Figure 3C). This result suggests that P granules are not prevented from moving with cortical flow by any such trap.

PAR-1 Functions to Stabilize P Granules that Reach the Posterior Cortex

PAR-1 might instead function to asymmetrically localize P granules by selectively stabilizing only those P gran-

ules that reach the PAR-1 domain at the posterior cortex. This would be consistent with a report that P granule epitopes are missing in late one-cell and two-cell embryos in *par-1* mutants [40] and might explain why P granules that do not reach the posterior cortex normally disappear near the end of the period of flow [20]. We tested whether P granules moved posteriorly but then disappeared in the absence of *par-1* by simultaneous DIC and fluorescence imaging of transgenic GFP::PGL-1, *par-1(RNAi)* embryos. P granules did move posteriorly during the period of cytoplasmic flow and then disappeared by the time flow ended (Figure 6A and Movie 3). During the period of movement, P granules moved in concert with neighboring yolk granules and vesicles (data not shown), as in wild-type embryos. The loss of P granules in *par-1(RNAi)* embryos occurred at a time similar to that when most of the P granules not localized to the posterior cortex disappear in wild-type embryos—during the period of flow and soon after flow ends (Figure 6; [20]). We have found that PGL-1 and other P granule components are affected similarly by the loss of PAR-1; antibodies to GLH-1 [41] and four antibodies that recognize unidentified P granule components (L123, L271, L416, PIF4; [42] and S.S., unpublished data) all failed to detect P granules in *par-1(it60)* embryos between the end of the period of flow and the two-cell stage, whereas they did detect P granules in wild-type embryos at the same stages and at other stages in *par-1(it60)* (data not shown). We conclude that PAR-1 is necessary to stabilize P granules against either disassembly or degradation during the period of flow.

If PAR-1 functions in P granule localization by locally stabilizing P granules that reach the posterior cortex during flow, then ectopically localizing PAR-1 throughout the embryo, even in a background that has cortical and central cytoplasmic flow, might be expected to result in many ectopically stabilized P granules. We first determined whether any of twelve existing *par-1* mutant alleles result in mislocalized but wild-type levels of PAR-1 protein, but no allele did (Supplemental Table 2). Many alleles resulted in little or no PAR-1 protein at the one- and two-cell stages and few or no P granules, consistent with the proposal that PAR-1 functions to stabilize P granules. Results also confirmed a previous report that in two alleles predicted to lack kinase activity, *it51* and *it90*, P granules are absent, but PAR-1 is enriched in the posterior cortex as in wild-type embryos [40], suggesting that PAR-1's kinase activity is essential to stabilize P granules at the posterior cortex.

Because none of the existing *par-1* alleles we examined resulted in significant levels of mislocalized PAR-1 protein, we sought other methods to mislocalize PAR-1 in a background that has cortical and central cytoplasmic flow. PAR-2 and PAR-3 are required to localize PAR-1 to the posterior cortex, and reducing *par-2* or *par-3*'s function by RNAi often results in an apparently hypomorphic phenotype in which cortical and central cytoplasmic flow occurs either as in the wild-type or for a shorter duration (J.-C. Labbé, personal communication, and R.J.C. and B.G., unpublished), [34, 43]. We therefore injected *par-2* or *par-3* double-stranded RNA (dsRNA) into GFP::PGL-1 transgenic animals. Examination of P granule dynamics in these backgrounds revealed that P granules move when cortical and central cytoplasmic

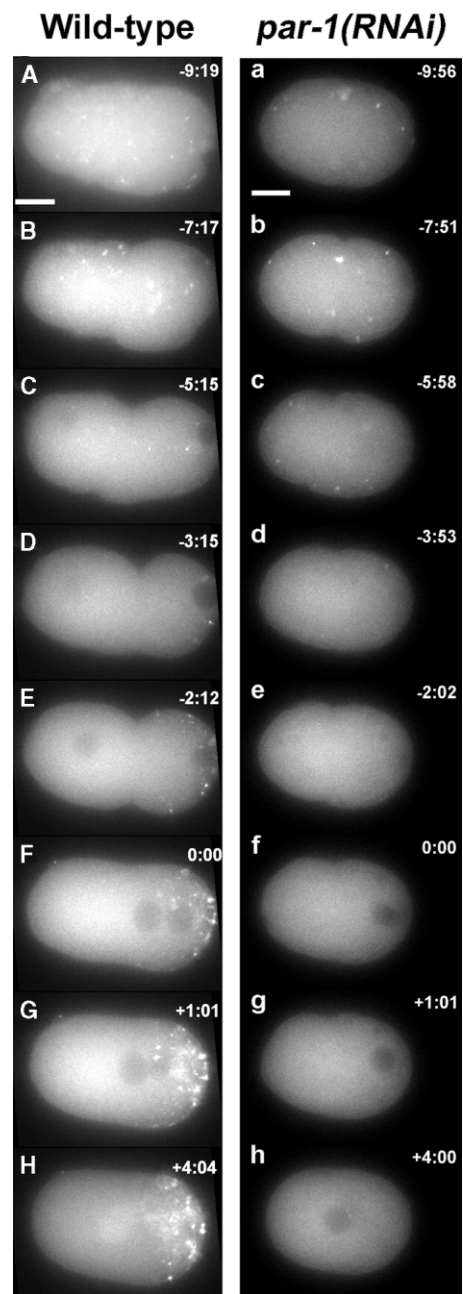


Figure 6. P Granules Move Posteriorly but Are Destabilized in the Absence of PAR-1

(A–H and a–h) Fluorescence recordings of the central plane of GFP::PGL-1 embryos from wild-type worms (left) and *par-1(RNAi)* worms (right). P granules move posteriorly and disappear in *par-1(RNAi)* embryos. Cortical planes showed similar results (data not shown). Time is shown in minutes and seconds before or after pronuclear meeting. Embryos were recorded every 12 s; selected frames are shown. The scale bars represent 10 μ m. See also Movies 2 and 3.

flow occurs, and most P granules end up in the posterior half of the embryo, but many are retained in areas of the embryo other than the posterior cell cortex (Figure 7, Figure S2). To determine if the stabilization of these P granules depends on PAR-1, we coinjected *par-1* dsRNA with *par-2* or *par-3* dsRNA into GFP::PGL-1

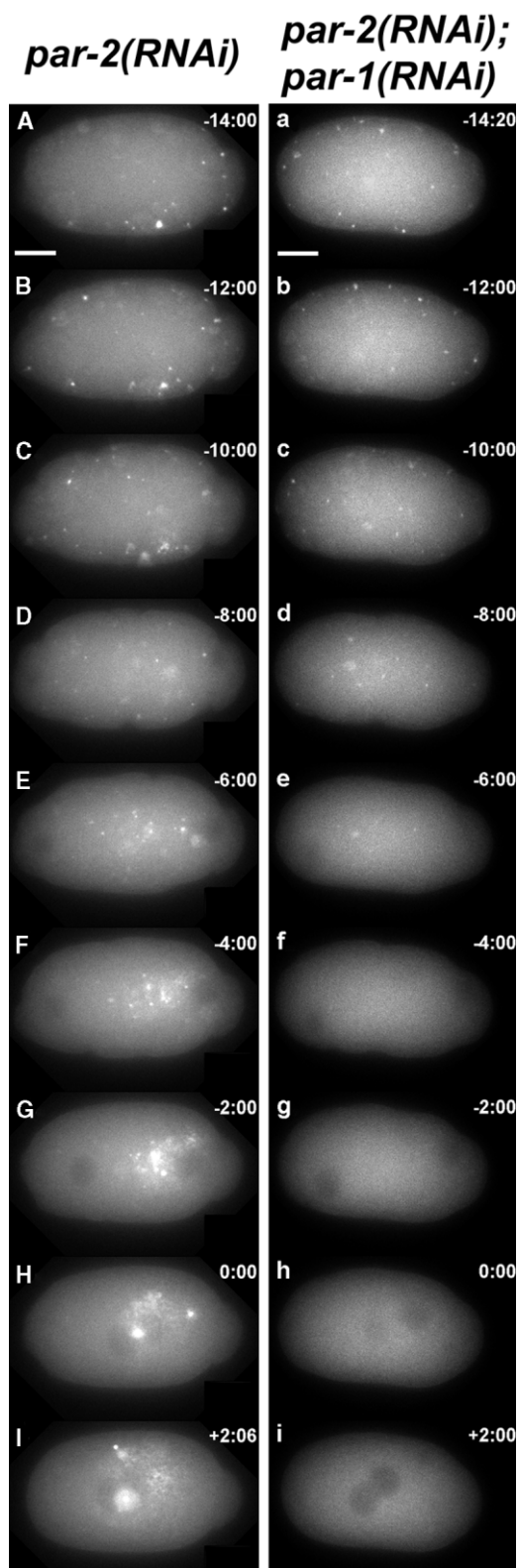


Figure 7. Ectopic Stabilization of P Granules in *par-2(RNAi)* Depends on PAR-1

(A–I and a–i) Fluorescence recordings of the central plane of GFP::PGL-1 embryos from *par-2(RNAi)* worms (left) and *par-2(RNAi); par-1(RNAi)* worms (right). P granules disappear in *par-2(RNAi); par-1(RNAi)* embryos. Cortical planes showed similar results (data not shown). Time

transgenic animals and recorded the resulting embryos. P granules disappeared during or just after the period of flow in these embryos (Figure 7, Figure S2). We conclude that mislocalized PAR-1 can ectopically stabilize P granules. Because we found that most P granules generally end up in the posterior half of the embryo even when PAR-1 is mislocalized, we infer that some P granule localization can occur even when PAR-1 is not localized and hence that PAR-1's normal role is to refine P granule localization rather than to act as the sole determinant of P granule localization.

PAR-1 is known to play a role in excluding MEX-5/6, proteins implicated in P granule degradation, from the posterior half of the zygote [28]. We therefore asked whether PAR-1 stabilizes P granules by excluding MEX-5/6 from the posterior. Loss of *mex-5/6* resulted in P granules being retained at the two-cell stage whether or not PAR-1 protein was present (Figure S3), suggesting that PAR-1 does protect P granules at least in part by excluding MEX-5/6 from the posterior half of the embryo.

Discussion

To understand how PAR proteins function to generate cell polarity, we have taken advantage of the potential to combine modern live-cell imaging techniques with an analysis of mutants in the *C. elegans* embryo. Our results, together with previous data, suggest a model in which PAR proteins establish polarity by two distinct mechanisms (Figure 8). First, PAR-2, -3, -4, and -6 and MEX-5/6 establish polarity by generating an actomyosin-based movement of the cortex away from the point of sperm entry. This movement generates two distinct cortical domains—a domain of new cortex with which PAR-2 dynamically associates in the posterior of the embryo and a domain of old cell cortex with which PAR-6 dynamically associates in the anterior of the embryo. This movement of the actin cortex to the anterior may drive the opposing flow of central cytoplasm and carry most of the P granules, which are enriched in the central cytoplasm after the beginning of flow, to the posterior. These movements do not result in the complete circulation of cortical and central cytoplasmic components, because the extent of cortical and central cytoplasmic flow is less than the full length of the embryo. Second, around the time that these movements stop, PAR-1, localized to the posterior cell cortex, refines the pattern of P granule localization by stabilizing only those P granules that have reached the posterior cell cortex.

A Mechanism for Generation of Cell Polarity by PAR Proteins

Cuenca et al. [22] have proposed that cell polarization in the *C. elegans* zygote proceeds by distinct establishment and maintenance phases. Our results suggest a mechanism by which cell polarization is established—by movement of the actin cortex and of cortical domains

is shown in minutes and seconds before or after pronuclear meeting. Embryos were recorded every 12 s; selected frames are shown. The scale bars represent 10 μ m. See also Movies 4 and 5.

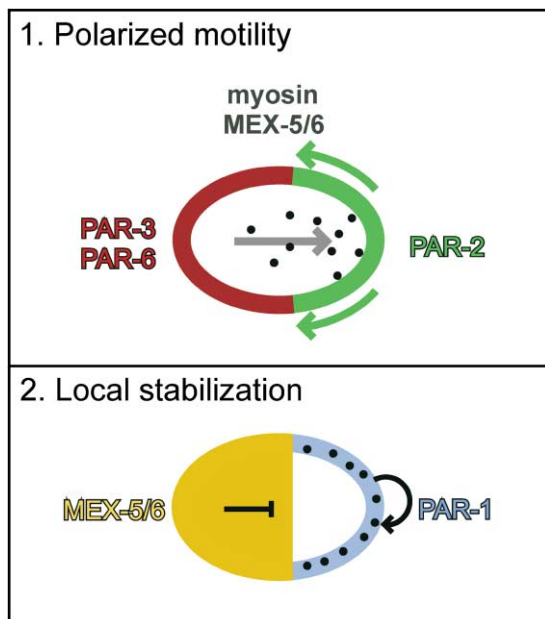


Figure 8. Two PAR-Dependent Mechanisms for Generating Asymmetries in the *C. elegans* Zygote

First (top diagram), cortical and central cytoplasmic flow (arrows), which is dependent on all of the proteins shown and some others not shown, results in the posterior movement of most P granules (black dots) and generates distinct cortical domains to which specific PAR proteins associate. Second (bottom diagram), PAR-1 stabilizes (black arrow) P granules that reach the posterior cortex, at least in part by excluding MEX-5/6, which destabilize P granules in the anterior half of the embryo. See text for further details and references.

to which PAR proteins associate and by movement of central cytoplasm and P granules in the opposite direction. PAR-2 may be involved in both this establishment phase and in a second, maintenance phase of cell polarization because PAR-2 is required for the full extent of cortical and central cytoplasmic flow (our results) but is also required to later exclude anterior PAR proteins from the posterior cell cortex after pronuclear meeting [22].

The loss of cytoplasmic flow in many of the *C. elegans* *par* mutants may, in large part, explain their mutant phenotypes. For example, we found that loss of the posterior cortical protein PAR-2 results in a partial failure of cortical flow. This would be expected to result in the generation of little new cortex in the posterior; consistent with this, anterior PAR proteins associate with most of the cell cortex in *par-2* mutants [43, 38]. The small amount of cytoplasmic flow in *par-2* mutants probably results in the incomplete localization of P granules previously observed in *par-2* mutants [39]. Mislocalized PAR-1 ectopically stabilizes these P granules (Figure 7).

Likewise, for anterior PAR proteins such as PAR-3 or PAR-6, loss of function results in a symmetric P granule distribution [39], most likely because the cytoplasmic flow that carries P granules posteriorly fails and because a resulting uniform distribution of PAR-1 [6] stabilizes P granules in ectopic locations. The global distribution of posterior PAR proteins in these backgrounds suggests that PAR-2 is normally prevented from associating

with old cortex by anterior PAR proteins [6]. Our findings show that the globally cortical protein PAR-4 functions in the same intracellular motility events as do some of the anteriorly or posteriorly localized PAR proteins. We have not extensively analyzed embryos that lack the globally cortical protein PAR-5 because null alleles of *par-5* do not yet exist, but preliminary recordings demonstrated a partially penetrant phenotype in which cortical and central cytoplasmic flow failed to occur (R.J.C. and B.G., unpublished data).

The *par* mutant phenotypes resemble those produced by loss of actomyosin contraction regulators such as the myosin II subunits NMY-2 and MLC-4 and loss of the actin binding protein POD-1, both of which result in the failure of cortical and central cytoplasmic flow [10, 44]. It has been proposed that actomyosin-based movement of the cell cortex in *C. elegans* and in other systems may be initiated and/or maintained by astral microtubules (see [13] for review). Although we have found that PAR proteins regulate microtubule dynamics after the period of flow [45], no defects in astral microtubule distributions before the period of flow have been reported in *C. elegans* *par* mutants, suggesting that PAR proteins probably function in the cortical response to astral microtubules. PAR proteins might modify the actin cortex in a manner that allows the cortex to move, perhaps by allowing local depolymerization of the contractile actomyosin mesh at the posterior pole. Alternatively, because a small amount of flow could be seen in many of the *par* mutants, it is possible that PAR proteins modify the cortex in a way that allows further flow propagation to be initiated by astral microtubules, independently of the PAR proteins.

The mechanisms by which PAR proteins drive cortical and central cytoplasmic flow are not yet clear. CDC-42, which associates with the PAR-3/PAR-6/PKC-3 complex in *C. elegans* and in other organisms, has well-characterized roles in modulating the actin cytoskeleton (reviewed in [16]). CDC-42 can induce actin polymerization via WASP and Arp2/3 in other systems [46]. If actin polymerization drives cortical motility as it has been proposed to do in migrating cells (see [12] for review), one would expect it to do so in the posterior of the embryo to drive cortical flow anteriorly. However, CDC-42 may function primarily in the anterior of the *C. elegans* embryo because *cdc-42(RNAi)* embryos have phenotypes that generally resemble loss of anterior PAR proteins instead of loss of posterior PAR proteins [47, 48]. How then might CDC-42 function in the anterior? Depolymerization of the actin meshwork near the astral microtubules in the posterior, along with a higher myosin contractility in the receding old cortex than in new cortex, may drive cortical flow [49, 11], and there is precedence for CDC-42 regulating myosin II activity: in a variety of systems, CDC-42 activates p21-activated kinases, and p21-activated kinases can upregulate myosin II activity by phosphorylating myosin light-chain kinase [50].

Although our results suggest a general mechanism by which a cell can produce two distinct cortical domains, it is not clear how specific PAR proteins recognize new or old cortical domains. PAR-2 associates with the cell cortex before fertilization, and even in gonads before oocytes are cellularized [34], whereas PAR-3 and PAR-6

are not cortically enriched until the time of meiosis [43, 38]. Therefore, PAR-6 does not associate preferentially with old cortex simply as a result of associating with cortex earlier. Instead, it appears that PAR-2 is specifically excluded from the cortex during the period in which PAR-3 and PAR-6 first associate with the cortex; PAR-2 enrichment at the cell cortex has been reported to decrease as oocytes mature [34]. The conclusion that PAR-2 is specifically excluded from the cortex by a PAR-3- and PAR-6-independent mechanism is supported by the dynamics of PAR-2 association with the cortex. As cortical flow begins, PAR-2 does not immediately associate with new cortex but instead does so with a 3–4 min delay. FRAP experiments on GFP::PAR-2 suggest that PAR-2 associates with the cell cortex far too dynamically to account for this 3–4 min delay on the basis of PAR-2 protein diffusion dynamics alone. Whether the cortex is modified or PAR-2 is modified at this time is not clear, but PAR-2 diffusion does not change significantly over time; PAR-2 is equally dynamic in the variable and transient anterior cap seen by Boyd et al. [34] soon after fertilization, in the expanding posterior cap discussed in this paper and at the two- and four-cell stages (R.J.C. and B.G., unpublished).

Generation of a Distinction between Soma and Germline

Our results on P granule localization are consistent with the classic studies of Hird et al. [20] and suggest specific roles for molecules involved in P granule localization. Hird et al. [20] reported that P granules become localized to one side of the *C. elegans* zygote in two ways. P granules localize first by moving toward the posterior. We have found that several PAR proteins are required for the cytoplasmic flow that appears to carry P granules. Second, P granules that do not reach the posterior disappear. We have found that PAR-1 is responsible for stabilizing those P granules that reach the posterior cortex. It is not yet known whether P granules disappear in the absence of *par-1* because they are disassembled or because individual P granule components are degraded. P granules reappear at the four-cell stage in *par-1* mutants ([40] and our unpublished data), suggesting that PAR-1 is no longer required to stabilize P granules at this stage and that if P granule components are degraded, they must be resynthesized very quickly.

P granules become enriched in the central cytoplasm near the time that cortical and central cytoplasmic flow begins. This may be essential for cortical and central cytoplasmic flow to result in an asymmetric pattern of P granules. Very little is known about the mechanisms by which this central cytoplasmic enrichment occurs, except that by live microscopic imaging it appears to occur by degradation or disassembly rather than by movement of cortical P granules (our unpublished data). Also, it appears to depend on MBK-2, an activator of protein degradation, because cortical P granules remain stable in published films of P granule movements in *mbk-2(RNAi)* embryos [36].

Derenzo et al. [51] recently discovered a mechanism for degradation of germline proteins in the soma in *C. elegans* embryos: MEX-5/6 activates the cullin/ubiqui-

tin-dependent degradation of PIE-1 and certain other germline proteins in the anterior of the embryo. P granule degradation does not appear to be targeted by this cullin complex; loss of complex members results in ectopic PIE-1 stabilization but not ectopic P granule stabilization. Whether other cullin complexes function in P granule degradation is not yet clear. However, the mechanisms that degrade PIE-1 and P granules do have in common a requirement for the activator of protein degradation MBK-2 [36, 37].

Evolutionary Conservation of Mechanisms

PAR proteins have been implicated in cell polarization in *Drosophila* oocytes, epithelia, and neuronal stem cells; mammalian epithelia and astrocytes; and *Xenopus* oocytes (for review, see [4, 14–16]). Our results suggest a model by which the PAR proteins might polarize cells in these systems. In some of these systems, the small size of cells or their inaccessibility in living tissues makes examining movements of labeled proteins in mutants difficult. However, there are some indications that similar mechanisms act in at least some of these systems. PKC-3 homologs in both *Drosophila* and mammals associate with PAR-6 homologs and phosphorylate the cytoskeletal protein Lgl, which is necessary for the asymmetric localization of other proteins [52] and can interact with non-muscle myosin II [53, 54]. Whether Lgl controls a myosin-dependent reorganization of cortical and central cytoplasmic components in these systems is not known.

PAR-1's role in stabilizing germline ribonucleoprotein complexes may be part of a conserved mechanism by which these complexes are maintained only in the germline; *Drosophila* PAR-1 phosphorylates and stabilizes the germline protein Oskar, which is a polar granule component [19]. *C. elegans* PAR-1 appears to require its kinase activity to stabilize P granules during and just after the period of flow ([40] and this paper), although no kinase targets are yet known. Whether *C. elegans* PAR-1 directly phosphorylates P granule proteins is unknown, although it is clear that it does not phosphorylate an Oskar homolog because no protein resembling Oskar is predicted in the *C. elegans* genome or by the sequence from any organism outside the dipteran insects. It will be interesting to determine to what extent the mechanisms of PAR-dependent cell polarization have been conserved and to what extent they have been altered through evolution.

Supplemental Data

Supplemental Data, including Supplemental Experimental Procedures, three figures, and seven movies are available with this article online at <http://www.current-biology.com/cgi/content/full/14/10/851/DC1/>.

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