PP1 phosphatases control PAR-2 localization and polarity establishment in *C. elegans* embryos

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Cell polarity relies on the asymmetric distribution of the conserved PAR proteins, which is regulated by phosphorylation/dephosphorylation reactions. While the kinases involved have been well studied, the role of phosphatases remains poorly understood. In *Caenorhabditis elegans* zygotes, phosphorylation of the posterior PAR-2 protein by the atypical protein kinase PKC-3 inhibits PAR-2 cortical localization. Polarity establishment depends on loading of PAR-2 at the posterior cortex. We show that the PP1 phosphatases GSP-1 and GSP-2 are required for polarity establishment in embryos. We find that codepletion of GSP-1 and GSP-2 abrogates the cortical localization of PAR-2 and that GSP-1 and GSP-2 interact with PAR-2 via a PP1 docking motif in PAR-2. Mutating this motif in vivo, to prevent binding of PAR-2 to PP1, abolishes cortical localization of PAR-2, while optimizing this motif extends PAR-2 cortical localization. Our data suggest a model in which GSP-1/-2 counteracts PKC-3 phosphorylation of PAR-2, allowing its cortical localization at the posterior and polarization of the one-cell embryo.

**Introduction**

Cell polarity is a fundamental property of cells required for many aspects of cell and animal biology. In migrating cells, for example, a front–rear polarity regulates migration in response to chemokines or antigens (Llense and Etienne-Manneville, 2015); in stem cells, cell polarity is a prerequisite for asymmetric cell division (Santoro et al., 2016); and in epithelial cells, the apical–basal polarity axis is required to establish the barrier function of the epithelium (Riga et al., 2020; Rodriguez-Boulan and Macara, 2014; Roignot et al., 2013).

In many different cells, polarity is regulated by the conserved partitioning defective (PAR) proteins, which have been identified in *Caenorhabditis elegans* (Goldstein and Macara, 2007; Rose and Gόnczy, 2014). The *C. elegans* zygote is a powerful model system for investigating the mechanism of cell polarity establishment and maintenance. The one-cell *C. elegans* embryo is polarized along the anterior–posterior (A-P) axis, with the anterior PAR proteins (the PDZ proteins PAR-3 and PAR-6; the atypical protein kinase C PKC-3; and the small GTPase CDC-42, from now on referred to as anterior PARs) enriched at the cortex in the anterior half of the embryo, and the posterior PAR proteins (the ring finger protein PAR-2; the kinase PAR-1; the lethal giant larvae ortholog, LGL-1; and the CDC-42 GAP CHIN-1, referred to as posterior PARs) enriched at the posterior cortex (reviewed in Goehring, 2014; Lang and Munro, 2017). This polarization results in a first asymmetric cell division, giving origin to two cells, AB and P1, with different sizes and fates. The zygote polarizes in two distinct phases, establishment and maintenance (Cuenca et al., 2003). Just after fertilization, PAR-3, PAR-6, and PKC-3 are uniformly distributed at the cortex, whereas PAR-1 and PAR-2 are in the cytoplasm (Fig. 1 A). Protein kinase C (PKC-3) phosphorylates PAR-2 and PAR-1, inhibiting their cortical localization and polarity establishment (Folkmann and Seydoux, 2019; Hao et al., 2006; Motegi et al., 2011). Polarity establishment relies on two redundant pathways, both dependent on the centrosomes. Shortly after the fertilization, a gradient of the mitotic kinase Aurora A (AIR-1) from the centrosomes of the paternal pronucleus triggers a cortical flow away from the newly defined posterior pole (Kapoor and Kotak, 2019; Klinkert et al., 2019; Reich et al., 2019; Zhao et al., 2019). This initiates the segregation of the anterior PARs to the anterior side of the embryo and liberates the posterior pole, allowing the localization of PAR-2 (and PAR-1; Munro et al., 2004). A second, redundant pathway relies on centrosomes-emitting microtubules, which promote loading of PAR-2 in the posterior (Fig. 1 A; Motegi et al., 2011). PAR-2 recruits PAR-1, thereby excluding the anterior PARs via PAR-1-dependent phosphorylation of PAR-3 (Motegi et al., 2011). Once polarity is established, mutual antagonism between the anterior and posterior PAR proteins ensures polarity maintenance (reviewed in Gubieda et al., 2020; Rose and Gόnczy, 2014). PKC-3 phosphorylates PAR-2 and inhibits its membrane association (Hao et al., 2006). Despite the fact that centrosomal
microtubules can protect PAR-2 from PKC-3 phosphorylation and therefore promote PAR-2 membrane association, mutations of the PAR-2 microtubule binding sites delay but do not abolish posterior cortical loading of PAR-2 in presence of normal cortical flows (Motegi et al., 2011). On the contrary, when the PKC-3 phosphorylation sites in PAR-2 are mutated to mimic phosphorylation, PAR-2 localization at the posterior cortex is abrogated, resulting in a defect in polarity establishment (Hao et al., 2006). This suggests that PAR-2 phosphorylation by PKC-3 must be relieved to ensure PAR-2 posterior cortical localization and hence proper polarity establishment.

Here, we show that the PP1 phosphatase GSP-2 plays an important role in polarity establishment. GSP-2 depletion suppresses the lethality (as previously shown Fievet et al., 2013) and the polarity defects of a temperature-sensitive pkc-3 mutant, suggesting that GSP-2 antagonizes PKC-3 function. GSP-2 depletion also results in defects in PAR-2 cortical localization that are exacerbated by the depletion of the PP1 phosphatase GSP-1.
Consistent with a role of GSP-2 and GSP-1 in PAR-2 dephosphorylation, PAR-2 contains a PPI binding motif, which we show is required for the interaction with GSP-1 and GSP-2 in two-hybrid assays. Mutations of this site known to abolish PPI binding abrogate PAR-2 cortical localization and polarity establishment in vivo. On the contrary, mutations that optimize the PPI binding motif extend PAR-2 cortical localization.

Our work identifies the PPI phosphatases, GSP-2 and GSP-1, as critical regulators of PAR-2 cortical localization and polarity establishment in the C. elegans embryo.

**Results**

**Depletion of GSP-2 suppresses the lethality and polarity phenotypes of a temperature sensitive pck-3 mutant**

GSP-2 has been identified as a suppressor of the embryonic lethality caused by a temperature-sensitive mutant allele of pck-3 (pck-3(ne4246); Fieve et al., 2013). PKC-3 phosphorylates PAR-2 and inhibits its localization at the anterior cortex (Hao et al., 2006; Motegi et al., 2011). In the pck-3(ne4246) strain, at 25°C, PAR-2 occupies the entire cortex (Rodriguez et al., 2017), resulting in polarity defects and embryonic lethality. We, therefore, asked whether depletion of GSP-2 was able to rescue the aberrant localization of PAR-2 observed in the pck-3(ne4246) embryos. Embryonic lethality of the pck-3(ne4246); gfp::par-2; ctrlRNAi strain at 24°C (the semirestrictive temperature at which PKC-3 is still partially active; see Materials and methods) was about 93.5% (Fig. 1 B). Consistent with the high embryonic lethality, embryos from pck-3(ne4246); gfp::par-2; ctrlRNAi mutant strain had impaired A-P polarity, with PAR-2 not being restricted anymore to the posterior, but distributed all around the cortex in one-cell stage embryos and partitioned symmetrically into the daughter AB and P1 cells (Fig. 1 C and Video 1).

Depletion of GSP-2 in gfp::par-2 worms did not result in embryonic lethality, and PAR-2 was localized at the anterior cortex (Fig. 1 B and C). Depletion of GSP-2 in the pck-3(ne4246); gfp::par-2 mutant rescued the embryonic lethality (Fig. 1 B). We found that PAR-2 localization was restored at the posterior cortex in one-cell stage embryos and was restricted to the P1 blastomere in two-cell stage embryos (Fig. 1 C and Video 1). Therefore, depletion of GSP-2 in the pck-3(ne4246); gfp::par-2 rescued embryonic lethality and PAR-2 localization defects of early embryos.

Polarity controls the posterior positioning of the mitotic spindle leading to a more posterior cleavage and to two-cell embryos with a bigger anterior cell (AB) and a smaller posterior cell (P1). Depletion of GSP-2 in the gfp::par-2 control strain resulted in a higher AB/P1 ratio compared to ctrlRNAi (Fig. S1 A).

In the pck-3(ne4246); gfp::par-2; ctrlRNAi, the cleavage furrow was shifted more toward the anterior, resulting in a reduced size asymmetry of the daughters. This phenotype was also rescued by depletion of GSP-2 (Fig. S1 A).

The two-cell embryos of C. elegans divide asynchronously, with the anterior AB cell dividing about 2 min before P1. This asynchrony is lost in the pck-3 mutant. We, therefore, tested whether GSP-2 depletion was able to rescue this phenotype. AB divided roughly 2 min before P1 both in ctrlRNAi and in gsp-2(RNAi) embryos, whereas in the pck-3(ne4246) mutant allele, the division time of AB and P1 was not significantly different (Fig. 1 D and Video 2). Depletion of GSP-2 in the pck-3(ne4246) strain was able to restore the asynchrony between AB and P1 (Fig. 1 D and Video 2).

One mechanism behind the regulation of asynchrony is the polarity-dependent localization of the mitotic kinase Polo-Like Kinase 1 (PLK-1). In the one-cell embryo, PLK-1 becomes enriched in the anterior cytoplasm and, at division, it is preferentially segregated in AB. This enrichment in AB triggers the earlier division of this blastomere (Budirahardja and Gόnczy, 2008; Nishi et al., 2008; Rivers et al., 2008). We, therefore, investigated the localization of PLK-1. In gsp-2(RNAi) embryos, PLK-1 was enriched in the anterior AB cell as in ctrlRNAi embryos (Fig. S1 B). In the pck-3(ne4246) embryos, the AB/P1 ratio of PLK-1 levels was close to 1, consistent with what has been previously reported in absence of the anterior PARs (Budirahardja and Gόnczy, 2008; Nishi et al., 2008; Rivers et al., 2008). In pck-3(ne4246); gsp-2(RNAi) embryos, PLK-1 anterior enrichment was restored (Fig. S1 B).

Taken together, these results show that depletion of GSP-2 suppresses the embryonic lethality and the polarity defects of the pck-3(ne4246) mutant allele.

**GSP-2 antagonizes PKC-3 in the regulation of PAR-2 localization at the posterior cortex**

The results that GSP-2 depletion rescues the PAR-2 localization defects of pck-3(ne4246) mutant embryos suggest that GSP-2 and PKC-3 antagonize each other in the regulation of PAR-2 cortical localization. PKC-3 phosphorylates PAR-2, thereby inhibiting its membrane localization (Hao et al., 2006). Since GSP-2 is a phosphatase, one possibility is that GSP-2 dephosphorylates PAR-2 allowing its posterior cortical localization. If this was the case, depletion of GSP-2 in control embryos should result in a reduction of cortical PAR-2, as PAR-2 would not be dephosphorylated. When GSP-2 was depleted in gfp::par-2 embryos, PAR-2 was localized at the posterior cortex (Fig. 1 C and Fig. 2 A). However, the size of the PAR-2 domain was smaller (Fig. 2 A), indicating that GSP-2 contributes to the formation of a PAR-2 domain of the correct size.

C. elegans embryos express a second PPI catalytic subunit, GSP-1, which is 85% identical to GSP-2 in the amino acid sequence (Sassa et al., 2003). GSP-1 and GSP-2 localize uniformly throughout the cytoplasm, in the nucleus, and on kinetochores in embryos (Sassa et al., 2003; Kim et al., 2017; Mangal et al., 2018), and they partially overlap in function (Peel et al., 2017). We, therefore, investigated whether GSP-1 can compensate for the function of GSP-2 in promoting PAR-2 cortical localization in one-cell embryos. We first asked whether GSP-1 depletion rescued the embryonic lethality of pck-3(ne4246); gfp::par-2 worms. Depletion of GSP-1 in gfp::par-2 worms did not result in embryonic lethality, and its depletion in the pck-3(ne4246); gfp::par-2 mutant did not rescue the embryonic lethality of this strain (Fig. 2 B). Consistent with this, PAR-2 was detected uniformly at the cortex in both pck-3(ne4246); gfp::par-2; ctrlRNAi and pck-3(ne4246); gfp::par-2, gsp-1(RNAi) (Fig. S3 A and Video 3). In addition, embryos depleted of GSP-1 did
Figure 2. Codepletion of GSP-1 and GSP-2 interferes with PAR-2 cortical posterior localization. (A) Left: representative midsection frames of time-lapse imaging of gfp::par-2 zygotes at pronuclear meeting, comparing ctrl(RNAi) and gsp-2(RNAi). N = 4. Right: quantification of the gfp::par-2 size domain in live zygotes at pronuclear meeting. Numbers inside the bars indicate sample size. N = 4. Mean is shown and error bars indicate SD. The P values were determined using two-tailed unpaired Student’s t test. (B) Embryonic lethality of gfp::par-2 and pkc-3(ne4246); gfp::par-2 embryos after ctrl(RNAi) and gsp-1(RNAi). The
not show a smaller PAR-2 domain compared to ctrl(RNAi) embryos (Fig. S2 B). Depletion of GSP-1 was efficient and specific (Fig. S4, A and B). Therefore, in contrast to GSP-2 depletion, GSP-1 depletion did not suppress the embryonic lethality and polarity defects observed in the pck-3(ne4246) mutant allele and did not impair the size of the PAR-2 domain.

We then asked if codepletion of GSP-1 and GSP-2 in the gfp::par-2 embryos impaired PAR-2 localization. Consistent with the previous results, we could observe a small but significant reduction in the size of the PAR-2 domain in the ctrl(RNAi); gsp-2(RNAi) embryos, while the PAR-2 domain was not reduced in the ctrl(RNAi); gsp-1(RNAi) embryos (Fig. 2, C and D). The intensity of the PAR-2 domain did not change in the single depletion (Fig. 2 E). In the GSP-1/-2 depleted embryos, we observed two phenotypes: in the majority of the embryos PAR-2 was mostly cytoplasmic (class I, 78.6%), as shown by an almost flat intensity profile, whereas a smaller percentage of embryos (class II, 21.4%) showed a weak PAR-2 localization at the posterior cortex (Fig. 2, C–E; and Video 4).

To conclude, codepletion of GSP-1 and GSP-2 results in a defect in PAR-2 posterior cortical localization. In addition, our data suggest that GSP-2 has a leading role in the regulation of PAR-2 localization, but GSP-1 can compensate in the absence of GSP-2.

GSP-1 and GSP-2 interact with PAR-2

GSP-2 and a PAR-2 N-terminal fragment (Fig. 3 A) were identified as interactors in a large-scale two-hybrid screen (Koorman et al., 2016). The genetic and physical interactions suggest that GSP-2 (with GSP-1) is the phosphatase that counteracts PKC-3 phosphorylation of PAR-2. We, therefore, tested whether GSP-2 and GSP-1 interacted with PAR-2. As previously shown (Koorman et al., 2016), we confirmed that GSP-2 interacted with PAR-2 (Fig. 3 B). Consistent with the redundancy in regulating PAR-2 localization, GSP-1 also interacted with PAR-2 (Fig. 3 B).

Analysis of the PAR-2 amino acid sequence revealed the presence of a degenerate PPI docking motif (164 RLFF 167) in the N-terminus of PAR-2, conserved in closely related nematode species (Fig. 3 A, upper panel, and Fig. 3 C). This suggests that PAR-2 may physically interact with PPI through this motif (Egloff et al., 1997; Hendrickx et al., 2009; Wakula et al., 2003; Zhao and Lee, 1997). To assess if the PPI docking motif present in PAR-2 is required for the interaction with GSP-1 and GSP-2, we mutated two critical amino acid residues into alanine (referred to as PAR-2 [RAFA], Fig. 3 A, lower panel). These substitutions have previously been shown to interfere with the binding between PPI phosphatases and their substrates (Meiselbach et al., 2006; Moreira et al., 2019). Interestingly, neither GSP-1 nor GSP-2 could interact with PAR-2 in the yeast two-hybrid system when the PPI docking motif in PAR-2 was mutated (Fig. 3 B).

These data show that PAR-2 interacts with both GSP-2 and GSP-1 in the two-hybrid assay, and this interaction depends on a PPI docking motif in PAR-2.

Mutations in the PPI docking motif of PAR-2 result in polarity defects

We set out to assess whether cell polarity was impaired if the PPI docking motif in PAR-2 was mutated. We generated a strain in which the PPI RLFF motif was mutated to RAFA in the endogenous gfp::par-2 (referred as gfp::par-2[RAFA]). The homozygous mutant worms were viable but exhibited high embryonic lethality (95.4% ± 4.5 SEM), and the surviving progeny was sterile (Fig. S5 A and Materials and methods). Our control, referred to as gfp::par-2*, was a mixture of homozygous worms expressing wild-type gfp::par-2 from both alleles and heterozygous worms expressing wild-type gfp::par-2 from one allele and mutant gfp::par-2[RAFA] from the other allele. We could not detect any difference in cortical PAR-2 domain size and intensity in the gfp::par-2* population compared to the gfp::par-2 (Fig. S5, B and C; and Materials and methods).

The gfp::par-2[RAFA] mutant embryos displayed phenotypes consistent with the impairment of polarity establishment. PAR-2 remained mostly cytoplasmic (Fig. 4, A and B; and Video S6), consistent with the phenotype of embryos codepleted of both PPI catalytic subunits and similar to what has been shown with the phosphomimetic mutant of PAR-2, in which seven PKC-3 phosphorylation sites have been mutated to glutamic acid (Hao et al., 2006). The position of the cleavage furrow during the first cell division was variable and shifted toward the anterior, resulting in a more symmetric first cell division with AB and P1 of equal size (Fig. S5 D). The levels of GFP::PAR-2[RAFA] were not significantly different from GFP::PAR-2 (Fig. S5, E and F, and figure legend), indicating that this phenotype is not the result of reduced PAR-2.

In control embryos, the P1 spindle rotates to be oriented along the A-P axis, whereas the AB spindle is orthogonal to the A-P axis (Fig. 4 C). Mutations that interfere with the establishment of polarity can alter spindle orientation at the second division.
We found that in the gfp::par-2 (RAFA) mutant embryos, the P1 spindle failed to rotate, resulting in an irregular arrangement of cells at the four-cell stage (Fig. 4 C). Furthermore, the AB and P1 blastomeres divided synchronously (Fig. 4 D and Video 6). Therefore, mutation of the PP1 docking motif in the endogenous PAR-2 strongly reduced PAR-2 cortical localization and led to polarity defects similar to the ones observed in par-2 loss of function embryos (Cheng et al., 1995; Kemphues et al., 1988).

If the phosphatase PP1 antagonizes the kinase activity of PKC-3 on PAR-2, depletion of PKC-3 in the gfp::par-2 (RAFA) mutant strain should result in PAR-2 localizing around the entire cortex. pkc-3(RNAi); gfp::par-2 embryos displayed uniform PAR-2 cortical localization in all the embryos analyzed (Fig. 4, E and F). These results suggest that the interaction between PAR-2 and GSP-1/2 has a crucial role in the regulation of PAR-2 cortical localization and establishment of polarity.

Optimizing the PP1 binding motif of PAR-2 results in aberrant cortical localization

One of the hallmarks of the PP1 docking motif is the high degeneracy at key positions of the motif (Davey et al., 2015). The optimal PP1 docking motif is the RVxF sequence (Wakula et al., 2003), and the valine was shown in vitro to contribute to a stronger interaction between PP1 and its substrates (Meiselbach et al., 2006). The PP1 docking motif in PAR-2 presents a leucine at position 165 instead of the optimal valine (Fig. 3 A). We, therefore, asked if optimizing the PP1 docking motif by mutating leucine to valine in vivo would alter the cortical localization of PAR-2. We hypothesized that if this mutation improves the binding of PAR-2 to PP1 phosphatases, the PAR-2 cortical domain may be extended.

We generated a strain where leucine 165 in the PP1 docking motif was mutated to valine in the endogenous gfp::par-2 (referred as gfp::par-2(L165V)). The mutant strain did not show embryonic lethality compared to gfp::par-2 worms (0.31% ± 0.25 SEM vs. 0.17% ± 0.06 SEM respectively), and the progeny was fertile. All gfp::par-2(L165V) zygotes, analyzed before symmetry breaking, displayed PAR-2 localization uniformly around the entire cortex, a phenotype that was not observed in control embryos (Fig. 5 A). During pronuclei migration, we detected both an anterior and a posterior PAR-2 domain in all the zygotes analyzed, indicating that in this strain restriction of PAR-2 to the posterior cortex is impaired. The anterior PAR-2 domain remained in 32% of embryos in mitosis and 16% of two-cell embryos (Fig. 5 A and Video 7).

These results show that optimizing in vivo the PP1 binding motif of PAR-2 results in aberrant cortical localization of PAR-2.
Figure 4. The PP1 binding site in PAR-2 is required in vivo for PAR-2 cortical posterior localization. (A) Representative midsection frames of time-lapse imaging of gfp::par-2* (the symbol ° indicates a mixture between wild-type gfp::par-2 homozygous worms expressing wild-type gfp::par-2 and heterozygous worms expressing wild-type gfp::par-2 from one allele and mutant gfp::par-2[RAFA] from the other allele) and gfp::par-2[RAFA] embryos at pronuclear meeting (n = 16 and n = 17, respectively). N = 6. (B) PAR-2 line profile of live zygotes at pronuclear meeting: gfp::par-2*, n = 16, and gfp::par-2[RAFA], n = 17, N = 6. Mean is shown and error bars indicate SD. (C) The wild-type and gfp::par-2[RAFA] dividing two-cell stage embryos. The upper panel shows a schematic representation.
PAR-2 before and during polarity establishment. This localization is corrected in most but not all embryos at later stages, suggesting that other yet unknown mechanisms are involved in the restriction of PAR-2 at the posterior cortex.

Depletion of GSP-2 in a temperature-sensitive plk-1 mutant allele rescues aberrant PAR-2 localization

An anterior PAR-2 domain, similar to the one observed in the gfp::par-2○(L165V) mutant, has been reported in embryos in meiosis (Wallenfang and Seydoux, 2000) and in embryos where the activity of the mitotic kinases PLK-1 and AIN-1 has been reduced (Kapoor and Kotak, 2019; Klinkert et al., 2019; Noatynska et al., 2010; Reich et al., 2019; Schumacher et al., 1998). We asked whether the anterior PAR-2 domain observed in the plk-1(or683) embryos might depend on GSP-2. To test this, we used the temperature-sensitive mutant allele plk-1(or683). We stained plk-1(or683); ctrl(RNAi) embryos with PAR-2 antibodies and found that PAR-2 localized at both the anterior and posterior domains, whereas in the wild type; ctrl(RNAi) embryos only one PAR-2 domain was detected (Fig. 6). Depletion of GSP-2 in the plk-1(or683) embryos resulted in a significant reduction of embryos with the anterior PAR-2 domain (Fig. 6). In addition, the length of the PAR-2 domain in the gsp-2(RNAi); plk-1(or683) embryos was comparable to the PAR-2 domain in control embryos, indicating that reduction of PLK-1 activity can rescue the smaller PAR-2 domain caused by GSP-2 depletion (Fig. 6 B).

Therefore, the anterior PAR-2 domain in the plk-1(or683) mutant allele depends, directly or indirectly, on the GSP-2 phosphatase, and the shorter PAR-2 domain caused by GSP-2 depletion depends on PLK-1, suggesting a genetic interaction in polarity regulation between PLK-1 and GSP-2.

Discussion

Establishment of the A-P axis is an essential process to ensure asymmetric cell division and proper development of the C. elegans embryo. Here, we show that in the one-cell embryo, this process is regulated by PPI phosphatases. We find that the PPI phosphatases GSP-2 and GSP-1 are required for the loading of PAR-2 at the cortex. Mutations in the PPI binding motif of PAR-2, which abrogate the interaction with GSP-1 and GSP-2 in yeast two-hybrid assays, result, in vivo, in the inability of PAR-2 to properly localize at the cortex. Our data suggest a model in which GSP-2 and GSP-1 counterbalance the activity of PKC-3 in the early embryo, allowing PAR-2 posterior cortical localization (Fig. 7).

These data also support previous findings in the field. Establishment of cell polarity in the one-cell C. elegans embryos relies on cortical flows that displace anterior PARs, including PKC-3, from the posterior, allowing loading of PAR-2 and PAR-1 (Munro et al., 2004; Shelton et al., 1999). In addition to this pathway, binding of PAR-2 to astral microtubules protects PAR-2 from PKC-3 phosphorylation and promotes its posterior cortical localization, therefore contributing to polarity establishment (Motegi et al., 2011). However, abolishing binding of PAR-2 to microtubules does not abrogate polarity establishment in the presence of cortical flows while a PAR-2 mutant that mimics PKC-3 phosphorylation is unable to polarize the embryo (Hao et al., 2006; Motegi et al., 2011). This suggests that the major polarization pathway involving the flows needs the action of phosphatases to dephosphorylate PAR-2, as shown by our data.

The PPI phosphatase GSP-2 was previously shown to be a suppressor of the embryonic lethality of the pkc-3(ne4246) mutant allele (Fievet et al., 2013). We find that depletion of GSP-2 in the pkc-3(ne4246) allele also rescues the polarity-related defects observed in the pkc-3(ne4246) mutant alone. Depletion of GSP-2 in otherwise control strains does not result in major defects in polarity establishment, as it would have been expected if GSP-2 was the only phosphatase targeting PAR-2. Codepletion of GSP-1 does result in polarity establishment defects. Although the polarity phenotype is enhanced in absence of both GSP-1 and GSP-2, only GSP-2 depletion can rescue pkc-3(ne4246) embryonic lethality and polarity defects and result in a smaller size of the PAR-2 domain in one-cell embryos, suggesting that GSP-2 is the phosphatase that has a more important role in polarity regulation. This is reminiscent of previous work showing that GSP-1 and GSP-2 have a partially overlapping role in the regulation of centrosome amplification. Interestingly, in this process, GSP-1 plays a more important role (Peel et al., 2017).

We find that PAR-2 contains a PPI docking motif, a common motif used by PPI phosphatases to physically interact with their substrates (Egloff et al., 1997; Hendrickx et al., 2009; Wakula et al., 2003; Zhao and Lee, 1997). This motif is also present in PAR-2 of closely related nematode species, suggesting that dephosphorylation of PAR-2 by PPI is a conserved process in nematodes. In this context, it is interesting to note that in C. elegans embryos the ortholog of the lethal giant larvae protein, LGL-1, can partially compensate for the loss of PAR-2 (Beatty et al., 2010; Hoege et al., 2010). In flies, Lgl is also an important regulator of polarity (Su et al., 2012). In epithelial cells, Lgl is removed from the cortex during mitosis in an aPKC and Aurora A phosphorylation-dependent manner. Restoration of cortical localization of Lgl is essential to maintain cell polarity and tissue architecture and is regulated by a PPI phosphatase (Moreira et al., 2019), similar to what we observe for PAR-2 in C. elegans zygotes.
Figure 5. The L165V mutation in PAR-2 causes atypical PAR-2 cortical localization. (A) Representative midsection frames of time-lapse videos of *gfp::par-2* and *gfp::par-2*(L165V) embryos at different cell division stages (for embryos before pronuclear migration: n = 9 and n = 14, respectively, and the other embryo stages showed: n = 14 and n = 25, respectively). The percentage on the bottom left of each image indicates the percentage of embryos with an anterior PAR-2 domain, except for the top panel (before pronuclear migration), which indicates the percentage of embryos with PAR-2 around the entire cortex. N = 4.

(B) Quantification of the *gfp::par-2* intensity from midsection images of live one-cell stage embryos of the indicated genotype. *gfp::par-2*, n = 28, *gfp::par-2*(L165V), n = 28.

(C) Western analysis of the indicated GFP::PAR-2 and tubulin levels in the indicated cell extracts.
n = 36, N = 2. Imaging of the gfp::par-2, used as control, has been performed the same days as the gfp::par-2(rafA) imaging of Fig. S5 E, and it is therefore the same data. Mean is shown and error bars indicate SD. The P values were determined using two-tailed unpaired Student’s t test. (C) On the left is a representative Western blot of extracts from gfp::par-2 and gfp::par-2(l165v) embryos. Tubulin is used as a loading control. On the right, normalized ratio GFP::PAR-2/Tubulin of each experiment, N = 3. The P value (equal to 0.49 [ns]) was determined using two-tailed paired Student’s t test between the values of control and gfp::par-2(l165v) measurements. (D) Representative midsections of time-lapse imaging of gfp::par-2 and gfp::par-2(l165v) one-cell embryos at different cell division stages, ctrl(RNAi), n = 13 and gsp-2(RNAi), n = 16; N = 8; RNAi was performed by injection. For the top panel (before pronuclear migration) n = 6 embryos for ctrl(RNAi) and n = 8 embryos for gsp-2(RNAi). As in A, percentage on the bottom left of each embryo indicates the percentage of embryos with an anterior PAR-2 domain, with the exception of the top panel. ns, P > 0.05; *, P < 0.05. n = number of embryos analyzed; N = number of independent experiments. Source data are available for this figure. Source Data F5.

Consistent with the role of the PPI docking motif for the interaction with PAR-2 and the phenotype observed with the codepletion of both GSP-1 and GSP-2, mutations of the two important amino acids in the PPI docking motif (leucine165 to alanine and phenylalanine167 to alanine, gfp::par-2(rafa)) abrogate the interaction with PAR-2 in the yeast two-hybrid system; more importantly, in vivo, these mutations impair the localization of PAR-2 at the posterior cortex and embryo viability, resembling the par-2 loss-of-function phenotype, consistent with the fact that dephosphorylation of PAR-2 is required for polarity establishment (Hao et al., 2006; Fig. 4 and Fig. S5). The gfp::par-2(rafa) mutant showed two interesting features. First, embryos from gfp::par-2” mothers did not show any difference in PAR-2 cortical levels and PAR-2 domain size compared to embryos from homozygote wild-type worms. We speculate that thanks to the property of PAR-2 to form oligomers (Arata et al., 2016), mutant PAR-2 can form oligomers with wild-type PAR-2 and localize properly to the cortex. Second, we also noticed that the gfp::par-2(rafa) mutant shows a weak PAR-2 localization at the posterior cortex (Video 5). One possibility is that in this mutant, the microtubule redundant pathway protects some PAR-2 from phosphorylation by PKC-3, but this alone is not sufficient to ensure the proper establishment of polarity.

Based on previous in vitro studies (Meiselbach et al., 2006), we have also optimized the PPI binding motif in vivo. The leucine165 to valine mutant (gfp::par-2(l165v)) showed an aberrant PAR-2 localization around the entire cortex prior to polarity establishment. During polarity establishment and at later stages, some of the embryos displayed an anterior and a posterior PAR-2 domain. This phenotype is reminiscent of the phenotype observed in embryos where PLK-1 and AIR-1 have been depleted (Kapoor and Kotak, 2019; Klinkert et al., 2019; Noatynska et al., 2019; Reich et al., 2019; Schumacher et al., 1998). Aurora A can be an activator of Polo-like kinase (Macurek et al., 2008; Seki et al., 2008; Tavernier et al., 2015). PLK-1 in C. elegans embryos is enriched in the anterior cytoplasm (Satake et al., 2008; Nishi et al., 2008; Rivers et al., 2008). One possibility is that Aurora A–activated PLK-1 contributes to keeping the GSP-2 levels/activity low at the anterior so that PAR-2 remains cytoplasmic (Fig. 7 A). This is consistent with previous genetic data showing that PLK-1 depletion can rescue PAR-2 cortical localization in par-2 temperature-sensitive mutants (Noatynska et al., 2010) and with our current data that GSP-2 depletion in plk-1(orc683) reduces the number of embryos with an anterior PAR-2 domain. However, consistent with published data, we did not observe any asymmetry in the localization/levels of GSP-2 and GSP-1 (Fig. S2). PLK-1 may phosphorylate and inhibit GSP-2 activity directly (Fig. 7 A). Our efforts to mutagenize predicted PLK-1 phosphorylation sites of GSP-2 into nonphosphorylatable ones did not show any phenotype consistent with an upregulation of GSP-2 activity. GSP-1 and GSP-2 are catalytic subunits of the protein phosphatase PPI. Catalytic subunits bind to regulatory subunits and these, in turn, regulate substrate specificity and the activity of the holoenzyme (Aggen et al., 2000). Therefore, PLK-1 might act on the yet-to-be-identified regulatory subunits to control PPI activity.

Experiments and modeling have shown that a spatially segregated kinase and a uniform phosphatase activity can generate a protein gradient (Griffin et al., 2011). Therefore, the asymmetric localization and activity of PKC-3 in the one-cell embryo could be sufficient to explain PAR-2 posterior cortical enrichment, even in the absence of a GSP-2 activity gradient (Fig. 7 B). Further studies are needed to understand whether the genetic interaction that we observe between PLK-1 and GSP-2 reflects a regulation of PPI activity, direct or indirect, by PLK-1 that contributes to polarity establishment and maintenance.

Altogether, our data suggest a model in which the proper balance between the activity of PPI phosphatases and PKC-3 is crucial to properly establish cortical polarity in one-cell embryos.

materials and methods

strains

The C. elegans strains used in this work are listed in Table S1. Worms were maintained on Nematode Growth Medium (NGM) plates seeded with OP50 bacteria, using standard methods (Brenner, 1974). Thermosensitive strains, such as pck-3(ne4246), pck-3(ne4246); gfp::par-2, and plk-1(orc683), were maintained at 15°C. All the other strains were maintained at 22°C.

Mutant strains were generated using CRISPR/Cas9 technology as described in Arribere et al. (2014). Single guide RNAs, repair templates to generate the mutant, PCR primers used to detect and sequence the mutation, and enzymes used for the screening are listed in Tables S2, S3, and S4. The ZU316 (gfp::par-2(l165v)) was backcrossed three times. For the ZU297 (gfp::par-2[rafA]), two independent isolates were analyzed. The gfp::par-2(rafa) strain was kept as a heterozygote (gfp::par-2/gfp::par-2[rafA]). gfp::par-2(rafa) adult worms are viable but produce 100% dead progeny, indicating that this mutation is maternal effect lethal. We balanced the mutation using the sc1s(2023)(dpy s2170) [umls41] III balancer strain (CGC51). The sc1s(2023)(dpy s2170) [umls41] hermaphrodites were first crossed with N2 males. Heterozygote males for the balancer strain were next crossed to N2 hermaphrodites to detect and sequence the mutation.

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Figure 6. PLK-1 limits the activity of GSP-2. (A) Representative images of fixed embryos of the indicated genotypes during pronuclei migration and at pronuclei centration/rotation, stained with anti-tubulin and anti-PAR-2 antibodies. Tubulin is in cyan, PAR-2 in magenta. The number of embryos analyzed is shown on the right in the pie-chart graph. N = 6. (B) PAR-2 size domain quantification of one-cell stage images from fixed embryos of the indicated genotypes once polarity is established. The quantification has been performed in embryos shown in the figure, and only the posterior PAR-2 domain was considered in the quantification. The P values were determined using the two-way ANOVA Tukey’s multiple comparisons test. N = 6. ns, P > 0.05; ****, P < 0.0001. In A and B, RNAi was performed by feeding. N = number of independent experiments.
crossed with the heterozygotes gfp::par-2 (RAFA). Heterozygote worms express the myo-2p::mKate tag and look phenotypically wild type; homozygote wild-type worms are dumpies, whereas homozygote mutant worms do not express the myo-2p::mKate tag. The balanced strain was used in Fig. S5, E and F.

**RNA interference**

Clones from the Ahringer feeding library (Ahringer, 2006; Kamath et al., 2003) were used when available (see Table S5). For the control, in the injection experiments, we used the clone C06A6.2 previously found in the laboratory to have no effect on the early embryonic cell division and to be 100% viable (Bondaz et al., 2019). For GSP-2, a DNA fragment was amplified from cDNA using Gateway-compatible oligonucleotide primers for Gateway-based cloning into the final pDEST-L4440 vector (forward primer, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-GTGACGTGCACGGACAATAC-3′, reverse primer, 3′-GGGACCACTTTGTACAAAAAAGCAGGCT-GTGACGTGCACGGACAATAC-5′). To produce double-strand RNA (dsRNA) for injections, the Promega Ribomax RNA production system was used. The RNAi constructs for GSP-1 and GSP-2 are specific, as shown in Fig. S4.

dsRNA was injected in L4/young adults, which were incubated at 20°C; embryos from injected hermaphrodites were analyzed after 24–28 h (Fig. 5 D) and 18–20 h (Fig. 2, C–E; and Fig. 4, E and F) after injection.

For the depletion of PKC-3 in the gfp::par-2 (RAFA) mutant strain, which is embryonically lethal, L4/young adult worms (homozygote wild-type, heterozygote, or homozygote par-2 [RAFA mutant] were single on OP50 seeded-NGM plates, to lay a few eggs, and subsequently injected with pkc-3 dsRNA. Injected worms were transferred to a new OP50-seeded NGM plate. Homozygous mutant gfp::par-2 (RAFA) worms were recognized by looking at the progeny in the original plates of noninjected worms, which did not hatch, as homozygote gfp::par-2 (RAFA) worms only lay dead eggs. For the codepletion of GSP-1 and GSP-2, a mixture of 1:1 dsRNAs was injected (Fig. 2, C–E).

RNA interference by feeding was performed using plates with 1 mM IPTG for the pkc-3(ne4246), gfp::gsp-1 and mNG::gsp-2 experiments and 3 mM IPTG for the plk-1(or683) experiments. The empty L4440 vector was used as control. For the experiment in Fig. 6 (feeding of the plk-1(or683) strain and N2), L1 worms were incubated at 15°C until the adult stage.

**Live imaging of embryos**

Adult hermaphrodites were dissected on a coverslip into a drop of Egg Buffer (118 mM NaCl, 48 mM KCl, 2 mM CaCl2, 2 mM MgCl2, and 25 mM Hepes, pH 7.5). Embryos were mounted on a 3% agarose pad. Time-lapse recordings (frames captured every 10 s) were performed using a Nikon ECLIPSE Ni-U microscope equipped with a Nikon DS-U3 Digital Camera and a 60×/1.25 NA objective. For Fig. 1 D, a Leica DM6000 microscope, equipped with a DFC 360 FX camera and a 63×/1.4 NA objective, was used. Images of embryos were taken every 10 s. Imaging was performed at 22°C.

**Immunostaining of embryos and image acquisition**

For staining of embryos, 20 gravid hermaphrodites were dissected in a drop of M9 (86 mM NaCl, 42 mM Na2HPO4, 22 mM KH2PO4, and 1 mM MgSO4) on an epoxy slide square (Thermo Fisher Scientific), previously coated with 0.1% poly-L-lysine. A 22 × 40-mm coverslip was added crosswise on the slide to squash the embryos. The slides were transferred on a metal block on dry ice for at least 10 min. Afterward, the coverslip was removed before fixing for 20 min in methanol. Immunostaining was performed as described in Spilker et al. (2009). The slides were...
transferred to a solution of PBS plus 0.2% Tween 20 (PBST) and 1% BSA for 20 min. The slides were incubated with primary antibodies diluted in PBST with 1% BSA overnight at 4°C (Rabbit anti PLK-1 [1:500; Tavernier et al., 2015], Rabbit anti-PAR-2 [1:200; Labbé et al., 2006], and mouse anti-tubulin [%1:1,000; Sigma-Aldrich]). After two washes of 10 min each in PBST, slides were incubated for 45 min at 37°C with a solution containing secondary antibodies (4 μg/ml Alexa Fluor 488– and/or 568–coupled anti-rabbit or anti-mouse antibodies) and 1 μg/ml DAPI to visualize DNA. Slides were then washed two times for 10 min in PBST before mounting using Mowiol (Calbiochem, 475904; 0.2 M Tris, pH 8.5, and 2.5% 1,4-diazabicyclo[2.2.2]octane).

Images were acquired using a Nikon ECLIPSE Ni-U microscope, equipped with a Nikon DS-U3 Digital Camera, and using a 60x/1.25 NA objective. Imaging was performed at 22°C.

**Yeast two-hybrid assay**

The interaction between PAR-2 and GSP-1/-2 was assessed using a GAL4-based system (Gateway, Invitrogen) using the MAV203 yeast strain. Full-length cDNAs of GSP-1 and GSP-2 were fused to the GALA DNA binding domain (Bait plasmid). A PAR-2 (1-335) fragment, both wild-type and mutant (RAFA), was fused to the GAL4 activation domain (Prey plasmid). The PAR-2 wild-type and mutant fragments and the GSP-1 and GSP-2 full length were first cloned into the pDONR201 and subsequently transferred to the pDEST22 vector (GAL4AD) and pDEST32 (GAL4DBD), respectively, using Gateway technology. Mutations were inserted by Pfu site-directed mutagenesis. A list of plasmids and primers used for the Y2H is provided in Tables S6 and S7, respectively. Transformants were selected on synthetic-defined medium (lacking leucine and tryptophan) plates. The interactions were tested by spotting single colonies containing the desired plasmids on a medium lacking leucine, tryptophan, and histidine and containing 50 mM of 3AT (3-amino-1,2,3-triazole; Sigma-Aldrich). Pictures of the plates were taken using the Fusion FX6 EDGE Imaging System (Vilber) equipped with an Evo-6 Scientific Grade CCD camera.

**Embryonic lethality**

To count the embryonic lethality, young adult worms were singled onto NGM plates seeded with OP50 and incubated 24 h at 24°C (Fig. 1 B and Fig. 2 B) and 20°C (Fig. S3 A and for the gfp::par-2(L165V) mutant). After 24 h, the adult worms were removed and the plates were again incubated at 24 and 20°C, respectively for 24 h. The ratio between the unhatched embryos over the total F1 progeny (unhatched embryos and larvae) was used to calculate the percentage of embryonic lethality.

**Western blotting for PAR-2 levels**

Embryos obtained by hypochlorite treatment from three medium plates of adult worms were resuspended in SDS sample buffer and denatured for 5 min at 95°C. An equal amount of embryos (~7,000) were loaded onto a 10% SDS acrylamide gel, and Western blotting was performed according to standard procedures with ECL detection (Vilber, Fusion FX). Primary antibodies (anti-TUBULIN, 1/2,500, mouse [Sigma-Aldrich] and anti-GFP, 1/2,500, rabbit [Pines]) were incubated overnight at 4°C, and HRP-conjugated secondary antibodies appropriate for each primary antibody were incubated for 45 min at room temperature.

**PAR-2 level measurement from Western blotting (Fig. 5 C and Fig. S5 F)**

α-TUBULIN and α-GFP levels were measured by drawing a region of interest of equal size using Fiji Imagej, and the mean intensity was used for quantification. The mean intensity of an equal region of interest, placed below the protein of interest, was used for background subtraction.

**Image analysis and measurement**

**PAR-2 cortical intensity line profile (Fig. 2 E and Fig. 4 B)**

The line profile of cortical PAR-2 was measured in one-cell stage embryos at pronuclear meeting. A segment 5-pixel-wide and of constant length, centered at the posterior cortex of the embryo and positioned with an angle of 90°C to the cortex, was traced using Imagej software. For each embryo, the average of three segments (upper, center, and lower posterior cortex) was used for quantification. The segment length was normalized to 1 and the line profile of each embryo was normalized to the average of the value in the cytoplasm at position 0 of the segment traced.

**Cortical PAR-2 size measurement (Fig. 2, A and D, Fig. S3 B, Fig. S5 C, and Fig. 6 B)**

The size of cortical gfp::par-2 domain was determined by measuring the length of the PAR-2 domain normalized for the total perimeter of the embryo at the pronuclear meeting. The perimeter of the embryo and the length of the PAR-2 domain were traced manually by using Imagej software. The length of the PAR-2 domain is represented as a percentage of the total perimeter of the embryo.

**Cortical PAR-2 intensity (Fig. 4 F and Fig. S5 B)**

The mean intensity of cortical gfp::par-2 was determined by tracing a line 5 pixels wide along the posterior cortex at pronuclear meeting. The mean intensity of the posterior cytoplasm was obtained by making a square of a fixed area using Imagej software. For Fig. 4 F, the ratio between the PAR-2 intensity at the cortex over the one in the cytoplasm is plotted, whereas for Fig. S5 B the mean intensity of the cytoplasm was subtracted from the mean intensity of cortical PAR-2.

**Cell cycle timing measurement (Fig. 1 D and Fig. 4 D)**

Cell cycle length was measured from the onset of nuclear envelope breakdown (NEBD) in P0 to the onset of NEBD in AB and P1. NEBD was measured at the time of nuclear membrane disappearance.

**AB/P1 ratio measurement (Fig. S1 A)**

AB and P1 length were measured at the time of cytokinesis furrow’s ingression, and then the ratio AB/P1 was calculated. A value equal to 1 indicates symmetry, whereas a value >1 indicates wild-type asymmetry with AB bigger than P1.

**PLK-1 asymmetry measurement (Fig. S1 B)**

The area of the AB and P1 cells was determined manually by using Imagej software and the intensity of PLK-1 was measured.
For the nucleus intensity, a circle around the nucleus was drawn. The intensity of the nucleus was subtracted from the intensity of PLK-1 of each cell. PLK-1 intensity ratio was determined by dividing the mean of PLK-1 intensity in AB over the mean of PLK-1 intensity in P1. Value equal to 1 indicates symmetric localization with PLK-1 more enriched in AB compared to P1, whereas value >1 indicates wild-type asymmetry with PLK-1 more enriched in AB compared to P1.

**Cleavage furrow position measurement (Fig. S5 D)**
The total length of the embryo and the length from the anterior pole to the cleavage furrow were measured manually using ImageJ software. The cleavage furrow position was determined as the ratio of the length from the anterior pole to the cleavage furrow over the total length of the embryo.

**Intensity profile for mNG::gsp-2 and gfp::gsp-1 (Fig. S2, A and B)**
The mean intensity of cytoplasmic mNG::gsp-2 and gfp::gsp-1 was determined by tracing a line in the cytoplasm from the anterior (position 0) to the posterior (position 1) of the embryo using ImageJ software. The background was subtracted from each value.

**PAR-2 level measurement in live embryos (Fig. 5 B and Fig. S5 E)**
The area of the whole embryo was obtained by tracing an ellipsoid using the ImageJ software, and the mean intensity of PAR-2 was measured. The mean intensity of the background was subtracted from the mean intensity of PAR-2.

**Protein phosphatase motif identification**
The PPI docking motif was identified using the Eukaryotic Linear Motif resource for Functional Sites in Proteins (http://elm.eu.org).

**Statistical analysis**
Statistical analysis was performed using GraphPad Prism 9. Details regarding the statistical test, the sample size, the experiment number, and the meaning of error bars are provided for each experiment in the corresponding figure legend, in the results, and summarized in Tables S8 and S9. Data distribution was assumed to be normal, but this was not formally tested.

Significance was defined as: ns, P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

**Online supplemental material**
Fig. S1 describes cell size asymmetry and PLK-1 localization in pkc-3 temperature-sensitive mutant and in GSP-2 depleted embryos. Fig. S2 describes mNG::gsp-2 and gfp::gsp-1 cytoplasmic localization. Fig. S3 describes the phenotype of gsp-1(RNAi) embryos. Fig. S4 shows the specificity and efficiency of gsp-1(RNAi) and gsp-2(RNAi). Fig. S5 compares the phenotypes (lethality, PAR-2 intensity, and domain cell size) of gfp::par-2* and gfp::par-2(RAFA) embryos and shows the levels of PAR-2 in the gfp::par-2 and the gfp::par-2(RAFA) balanced strains. Videos 1 and 3 show the first division and gfp::par-2 localization of pkc-3(ne4246) and pkc-3(ne4246); gsp-2(RNAi) (Video 1) and pkc-3(ne4246); gsp-1(RNAi) (Video 3) embryos. Video 2 shows the first and second division of pkc-3(ne4246) and pkc-3(ne4246); gsp-2(RNAi) embryos. Video 4 shows the first and second division of gfp::par-2(RAFA) embryos and control embryos. Video 5 shows the PAR-2 localization in gfp::par-2(RAFA) embryos and control. Video 6 shows the first and second division of gfp::par-2(L65V) embryos and control. Table S1 shows the genotypes of strains used in this study. Tables S2, S3, and S4 show all the reagents used for CRISPR. Table S5 summarizes the clones used for RNA interference. Tables S6 and S7 summarize the plasmids used for the two-hybrid experiment. Tables S8 and S9 summarize the statistical analyses used in this study.

**Data availability**
All the strains and reagents generated in this study are available from the corresponding author upon request. All raw data associated with the experiments have been deposited in https://doi.org/10.26037/yareta.wrebka2l2vc4ddxmplelewshg6m.

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**References**
Aggen, J.B., A.C. Naim, and R. Chamberlin. 2000. Regulation of protein phosphatase-1. Chem. Biol. 7:R13–R23. https://doi.org/10.1016/s1074-5521(00)00069-7

Ahringer, J. 2006. Reverse Genetics. WormBook: The Online Review of C. elegans Biology [Internet], WormBook; 2005–2018. https://www.ncbi.nlm.nih.gov/books/NBK19711/
Arata, Y., M. Hiroshima, C.G. Pack, R. Ramanujam, F. Motegi, K. Nakazato, Y. Shindo, P.W. Wiseman, H. Sawa, T.J. Kobayashi, et al. 2016. Cortical polarity of the Drosophila protein PAR-2 is maintained by exchange rate kinetics at the cortical-cytoskeletal boundary. Cell Rep. 16:2186–2196. https://doi.org/10.1016/j.celrep.2016.07.047

Arribere, J.A., R.T. Bell, B.X.H. Fu, K.L. Artilles, P.S. Hartman, and A.Z. Fire. 2014. Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in Caenorhabditis elegans. Genetics. 198:837–846. https://doi.org/10.1534/genetics.114.167912

Beatty, A. D., Morton, and K. Kephues. 2010. The C. elegans homolog of Drosophila Lethal giant larval functions redundantly with PAR-2 to maintain polarity in the early embryo. Development. 137:3995–4004. https://doi.org/10.1242/dev.056028

Bondax, A., L. Cirillo, P. Meraldi, and M. Gotta. 2019. Cell polarity-dependent centrosome separation in the C. elegans embryo. J. Cell Biol. 218: 4112–4126. https://doi.org/10.1083/jcb.201902109

Brenner, S. 1974. The genetics of Caenorhabditis elegans. Genetics. 77:71–94. https://doi.org/10.12738/g77.1.71

Budirahardja, Y., and P. Gönçzy. 2008. PLK-1 asymmetry contributes to asynchronous cell division of C. elegans embryos. Development. 135: 1303–1310. https://doi.org/10.1242/dev.019127

Castiglioni, V.G., H.R. Pires, R. Rosas Bertolini, A. Riga, J. Kerver, and M. Boxem. 2020. Epidermal PAR-6 and PKC-3 are essential for larval development of C. elegans and organize non-centrosomal microtubules. Elife. 9:e62067. https://doi.org/10.7554/elif.e62067

Cheng, N.N., C.M. Kirby, and K.J. Kemphues. 2019. Identification of genes required for cytoplasmic localization in early C. elegans embryos. Cell. 183:311–320. https://doi.org/10.1016/j.cell.2019.02.019

Kim, T., P. Lara-Gonzales, B. Prevo, F. Mettinger, D.K. Cheerambathur, K. Oegema, and A. Desai. 2017. Kinetochores accelerate or delay APC/C activation by directing Cdc20 to opposing fates. Genes Dev. 31: 1089–1094. https://doi.org/10.1101/gad.302067.117

Klinkert, K., N. Levermann, F. Gross, C. Gentili, L. von Tobel, M. Pierron, C. Busso, S. Herrman, S.W. Grill, K. Kruse, and F. Gönczy. 2019. Aurora A depletion reveals centrosome-independent polarization mechanisms in Caenorhabditis elegans. Elife. 8:e44552. https://doi.org/10.7554/eLife.44552

Koorman, T., D. Klopstra, M. van der Voet, I. Lemmens, J.J. Ramalho, S. Nieuwenhuize, S. van den Heuvel, J. Tavernier, J. Nance, and M. Boxem. 2016. A combined binary interaction and phenotypic map of C. elegans cell polarity proteins. Nat. Cell Biol. 18:337–346. https://doi.org/10.1038/ncomms13300

Labbé, J.C., A. Pacquiot, T. Marty, and M. Gotta. 2006. A genomewide screen for suppressors of par-2 uncovers potential regulators of PAR protein-dependent cell polarity in Caenorhabditis elegans. Genetics. 174:285–295. https://doi.org/10.1534/g3.116.015017

Lang, C.P., and E. Munro. 2017. The PAR proteins: From molecular circuits to dynamic self-stabilizing cell polarity. Development. 144:3405–3416. https://doi.org/10.1242/dev.139063

Lense, F., and S. Etienne-Manneville. 2015. Front-to-Rear polarity in migrating cells. In Cell Polarity: Biological Role and Basic Mechanism. K. Ebnet, editor. Springer International Publishing, New York. 115–146. https://doi.org/10.1007/978-3-319-14634-5_4

Macurek, L., A. Lindqvist, D. Lim, M.A. Lampson, R. Klopmaeker, R. Freire, C. Clouin, S.S. Taylor, M.B. Yaffe, and R.H. Medema. 2008. Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery. Nature. 455:119–123. https://doi.org/10.1038/nature07185

Mangal, S., J. Sacher, T. Kim, D.S. Osório, F. Motegi, A.X. Carvalho, K. Oegema, and E. Zanin. 2018. TPX1-1 activates Aurora A to clear contractile ring components from the polar cortex during cytokinesis. J. Cell Biol. 217:837–848. https://doi.org/10.1083/jcb.201706021

Meiselbach, H., H. Stich, and R. Enz. 2006. Structural analysis of the protein phosphatase 1 docking motif: Molecular description of binding specificities identifies interacting proteins. Chem. Biol. 13:49–59. https://doi.org/10.1016/j.chembiol.2005.10.009

Moreira, S., M. Oswald, G. Ventura, M. Gonçalves, C.E. Sunkel, and E. Morais-de-Sá. 2019. PPI-Mediated dephosphorylation of Lgl controls apical-basal polarity. Cell Rep. 26:293–301. https://doi.org/10.1016/j.celrep.2018.12.060

Motegi, F., S. Zonies, Y. Hao, A.A. Cuenca, E. Griffin, and G. Seydoux. 2011. Microtubules induce self-organization of polarized PAR domains in Caenorhabditis elegans zygotes. Nat. Cell Biol. 13:1361–1367. https://doi.org/10.1038/ncb2354

Munro, E., J. Nance, and J.R. Priess. 2004. Cortical flows powered by asymmetrical contraction transport PAR proteins to establish and maintain anterior-posterior polarity in the early C. elegans embryo. Dev. Cell. 7: 413–424. https://doi.org/10.1016/j.devcel.2004.08.021

Nishi, Y., E. Rogers, S.M. Robertson, and R. Lin. 2008. Polo kinases regulate C. elegans embryonic polarity via binding to DYRK2-prized MEX-5 and MEX-6. Development. 135:687–697. https://doi.org/10.1242/dev.014325

Noatyńska, A., C. Panbianco, and M. Gotta. 2010. SPAT-1/Bora acts with Polo-like kinase 1 to regulate PAR polarity and cell cycle progression. Development. 137:3315–3325. https://doi.org/10.1242/dev.053293

Peel, N., J. Iyer, A. Naik, M.P. Dougan, M. Decker, and K.F. O’Connell. 2017. Protein phosphatase 1 down regulates 2YG-1 levels to limit centriole duplication. PLoS Genet. 13:e1006543. https://doi.org/10.1371/journal.pgen.1006543

Reich, J.D., L. Hubatsch, R. Illukkumbura, F. Peglön, T. Bland, N. Hirani, and N.W. Goehring. 2019. Regulated activation of the PAR polarity network engineer's specific and specific response to spatial cues. Curr. Biol. 29: 1911–1923.e5. https://doi.org/10.1016/j.cub.2019.04.058

Riga, A., V.G. Castiglioni, and M. Boxem. 2020. New insights into apical-basal polarization in epithelia. Curr. Opin. Cell Biol. 62:1–8. https://doi.org/10.1016/j.celbi.2019.07.017

Calvi et al. 2015. PP1 phosphatases regulate polarity in C. elegans. Journal of Cell Biology. 165:1083–1088. https://doi.org/10.1083/jcb.20201048
Rivers, D.M., S. Moreno, M. Abraham, and J. Ahringer. 2008. PAR proteins direct asymmetry of the cell cycle regulators Polo-like kinase and Cdc25. J. Cell Biol. 180:877–885. https://doi.org/10.1083/jcb.200710018

Rodriguez, J., F. Peglion, J. Martin, L. Hubatsch, J. Reich, N. Hirani, A.G. Gubieda, J. Roffey, A.R. Fernandes, D. St Johnston, et al. 2017. aPKC cycles between functionally distinct PAR protein assemblies to drive cell polarity. Dev. Cell. 42:400–415.e9. https://doi.org/10.1016/j.devcel.2017.07.007

Rodriguez-Boulan, E., and I.G. Macara. 2014. Organization and execution of the epithelial polarity programme. Nat. Rev. Mol. Cell Biol. 15:225–242. https://doi.org/10.1038/nrm3775

Roignot, J., X. Peng, and K. Mostov. 2013. Polarity in mammalian epithelial morphogenesis. Cold Spring Harbor Perspect. Biol. 5:a013789. https://doi.org/10.1101/cshperspect.a013789

Santoro, A., T. Vlachou, M. Carminati, P.G. Pelicci, and M. Mapelli. 2016. Molecular mechanisms of asymmetric divisions in mammmary stem cells. EMBO Rep. 17:1700–1720. https://doi.org/10.15252/embr.201643021

Sasa, T., H. Ueda-Ohba, K.i. Kitamura, S.i. Harada, and R. Hosono. 2003. Role of C. elegans protein phosphatase type 1, CeGLC-7 β, in metaphase to anaphase transition during embryonic development. Exp. Cell Res. 287:350–360. https://doi.org/10.1016/s0014-4827(03)00157-5

Schumacher, J.M., N. Ashcroft, P.J. Donovan, and A. Golden. 1998. A highly conserved centrosomal kinase, AIR-1, is required for accurate cell cycle progression and segregation of developmental factors in C. elegans embryos. Development. 125:4391–4402. https://doi.org/10.1242/dev.125.22.4391

Seki, A., J.A. Coppinger, C.Y. Jang, J.R. Yates, and G. Fang. 2008. Bora and the kinase Aurora cooperatively activate the kinase Plk1 and control mitotic entry. Science. 320:1655–1658. https://doi.org/10.1126/science.1157425

Shelton, C.A., J.C. Carter, G.C. Ellis, and B. Bowerman. 1999. The nonmuscle myosin regulatory light chain gene mlc-4 is required for cytokinesis, anterior-posterior polarity, and body morphology during C. elegans embryogenesis. J. Cell Biol. 146:439–451. https://doi.org/10.1083/jcb.146.2.439

Spilker, A.C., A. Rabilotta, C. Zbinden, J.C. Labbe, and M. Gotta. 2009. MAP kinase signaling antagonizes PAR-1 function during polarization of the early C. elegans embryo. Genetics. 183:965–977. https://doi.org/10.1534/genetics.109.106716

Su, W.H., D.D. Mruk, E.W.P. Wong, W.Y. Lui, and C.Y. Cheng. 2012. Polarity protein complex Scribble/Lgl/Dlg and epithelial cell barriers. Adv. Exp. Med. Biol. 763:149–170. https://doi.org/10.1007/978-1-4614-4711-5_7

Tavernier, N., A. Nootynska, C. Panbianco, L. Martino, L. Van Hove, F. Schwager, T. Leger, M. Gotta, and L. Pintard. 2015. Cdk1 phosphorylates SPAT-1/Bora to trigger PLK-1 activation and drive mitotic entry in C. elegans embryos. J. Cell Biol. 208:661–669. https://doi.org/10.1083/jcb.201408064

Wakula, P., M. Beullens, H. Ceulemans, W. Stalmans, and M. Bollen. 2003. Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. J. Biol. Chem. 278:18817–18823. https://doi.org/10.1074/jbc.M300752200

Wallenfang, M.R., and G. Seydoux. 2000. Polarization of the anterior-posterior axis of C. elegans is a microtubule-directed process. Nature. 408:89–92. https://doi.org/10.1038/35040562

Zhao, P., X. Teng, S.N. Tantririmudalige, M. Nishikawa, T. Wohland, Y. Toyama, and F. Motegi. 2019. Aurora-A breaks symmetry in contractile actomyosin networks independently of its role in centrosome maturation. Dev. Cell. 48:631–645.e6. https://doi.org/10.1016/j.devcel.2019.02.012

Zhao, S., and E.Y. Lee. 1997. A protein phosphatase-1-binding motif identified by the panning of a random peptide display library. J. Biol. Chem. 272:28368–28372. https://doi.org/10.1074/jbc.272.45.28368
Figure S1. \textit{gsp-2(RNAi)} rescues AB/P1 asymmetry and PLK-1 localization in the \textit{pkc-3(ne4246)} mutant allele. (A) Quantification of cell size asymmetry (AB/P1 ratio). AB/P1 ratio equal to 1 correspond to cell symmetry, values higher or lower than 1 correspond to cell asymmetry. Numbers inside the bars indicate sample size. \( N = 4 \). Mean is shown and error bars indicate SD. The \( P \) values were determined using two-way ANOVA Tukey’s multiple comparisons test. (B) Representative two-cell stage images of fixed embryos of the indicated genotypes. DNA (DAPI) is in magenta and PLK-1 in cyan. Right: quantification of the PLK-1 levels (AB/P1 ratio) in the two-cell stage fixed embryos. Numbers inside the bars indicate sample size. \( N = 2 \). Mean is shown and error bars indicate SD. The \( P \) values were determined using two-way ANOVA Tukey’s multiple comparisons test. ns, \( P > 0.05 \); **, \( P < 0.01 \); ****, \( P < 0.0001 \). Scale bars, 5 \( \mu \)m. Anterior is to the left and posterior to the right. In both A and B, RNAi was performed by feeding. \( N \) = number of independent experiments.
Figure S2. mNG::gsp-2 and gfp::gsp-1 localize uniformly in the cytoplasm. (A) Left: representative midsection images of mNG::gsp-2 at three different stages. Right: mNG::gsp-2 intensity profile before pronuclear migration \( (n = 9, \text{pink line}) \), during pronuclear migration \( (n = 15, \text{green line}) \) and at pronuclear meeting \( (n = 11, \text{orange line}) \). \( N = 2 \). The mean is shown and error bars indicate SD. (B) Left: representative midsection images of gfp::gsp-1 at three different stages. Right: gfp::gsp-1 intensity profile before pronuclear migration \( (n = 9) \), during pronuclear migration \( (n = 15) \), and pronuclear meeting \( (n = 11) \). \( N = 2 \). Mean is shown and error bars indicate SD. \( n \) = number of embryos analyzed; \( N \) = number of independent experiments. Scale bars, 5 \( \mu \text{m} \). Anterior is to the left and posterior to the right.
Figure S3. \textit{gsp-1(RNAi)} does not rescue PAR-2 localization in the \textit{pkc-3(ne4246); gfp::par-2} strain and does not affect PAR-2 size domain in the \textit{gfp::par-2} strain. (A) Representative midsection frames of time-lapse videos of \textit{gfp::par-2} and \textit{pkc-3(ne4246); gfp::par-2} one-cell embryos at pronuclear meeting, comparing ctrl\textit{(RNAi)} and \textit{gsp-1(RNAi)}. In \textit{gfp::par-2; ctrl(RNAi)} and \textit{gfp::par-2; gsp-1(RNAi)}, PAR-2 localizes at the posterior cortex (\(n=8\) and \(n=8\), respectively). In \textit{pkc-3(ne4246); gfp::par-2; ctrl(RNAi)} and \textit{pkc-3(ne4246); gfp::par-2; gsp-1(RNAi)}, PAR-2 signal is detected uniformly at the cortex (\(n=10\) and \(n=13\), respectively). \(N=3\). (B) Quantification of the \textit{gfp::par-2} size domain in live zygotes at pronuclear meeting, comparing ctrl\textit{(RNAi)} and \textit{gsp-1(RNAi)}. Numbers inside the bars indicate sample size. \(N=3\). The P values were determined using two-tailed unpaired Student’s t test. Mean is shown and error bars indicate SD. ns, \(P > 0.05\). Scale bars, 5 \(\mu\)m. Anterior is to the left and posterior to the right. In both A and B, RNAi was performed by feeding, \(n\) = number of embryos analyzed, \(N\) = number of independent experiments.
Figure S4.  

**gsp-1(RNAi)** and **gsp-2(RNAi)** are efficient and specific for GSP-1 and GSP-2, respectively.  

(A) Representative midsection images of the indicated genotypes.  

(B) Quantification of gfp::gsp-1 levels in ctrl(RNAi; n = 25), gsp-1(RNAi) (n = 23), and gsp-2(RNAi; n = 20). N = 2. Mean is shown and error bars indicate SD. The P values were determined using ordinary one-way ANOVA.  

(C) Representative midsection images of the indicated genotypes.  

(D) Quantification of mNG::gsp-2 levels in mNG::gsp-2 after ctrl(RNAi; n = 18), gsp-2(RNAi) (n = 20), and gsp-1(RNAi) (n = 20). N = 2. Mean is shown and error bars indicate SD. The P values were determined using ordinary one-way ANOVA. RNAi was performed by feeding in all experiments shown in this figure. n = number of embryos analyzed; N = number of independent experiments. Scale bars, 5 µm. Anterior is to the left and posterior to the right. ns, P > 0.05; ****, P < 0.0001.
Figure S5. **The PP1 motif in PAR-2 is crucial for proper cell polarity.** (A) Embryonic lethality of gfp::par-2° (refer also later to a homozygous worms expressing wild-type gfp::par-2 and heterozygous worms expressing wild-type gfp::par-2 from one allele and mutant gfp::par-2(ARFA) from the other allele) and gfp::par-2(ARFA) homozygous mutant strain. The reported values correspond to the percentage of unhatched embryos over the total progeny (larvae and unhatched embryo). N = 4. Mean is shown and error bars indicate SEM. The P values were determined using two-tailed unpaired Student’s t test. (B) Measurement of the PAR-2 cortical intensity in the wild-type gfp::par-2 and in the gfp::par-2° embryos at pronuclear meeting. Numbers inside the bars indicate sample size. Mean is shown and error bars indicate SD. The P values were determined using two-tailed unpaired Student’s t test. (C) Quantification of the PAR-2 size domain in live zygotes at pronuclear meeting, comparing gfp::par-2 and gfp::par-2°. Numbers inside the bars indicate sample size. N = 2. Mean is shown and error bars indicate SD. The P values were determined using two-tailed unpaired Student’s t test. (D) Quantification of the cleavage furrow position in gfp::par-2° and gfp::par-2(ARFA). gfp::par-2(ARFA) mutant show cleavage furrow position more toward the anterior compared with gfp::par-2° (n = 23 and n = 17, respectively). N = 6. Mean is shown and error bars indicate SD. The P values were determined using two-tailed unpaired Student’s t test. (E) Quantification of the PAR-2 intensity from midsection images of live one-cell stage embryos of the indicated genotype. N = 2. Mean is shown and error bars indicate SD. The P values were determined using two-tailed unpaired Student’s t test. (F) On the left, representative Western blot of embryo extract, showing level of GFP::PAR-2 and TUBULIN, which is used as a loading control, for both gfp::par-2 and gfp::par-2(ARFA). On the right, normalized ratio GFP::PAR-2/TUBULIN. N = 3. The P value (equal to 0.1871 [ns]) was determined using two-tailed paired Student’s t test between the values of gfp::par-2 and gfp::par-2(ARFA). n = number of embryos analyzed, N = number of independent experiments. Scale bars, 5 µm. Anterior is to the left and posterior to the right. ns, P > 0.05; ****, P < 0.0001. Source data are available for this figure: SourceData FS5.
PP1 phosphatases regulate polarity in *C. elegans*

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**Video 1.** *gsp-2(RNAi)* rescues PAR-2 posterior cortical localization in the *pkc-3(ne4246); gfp::par-2* embryos. *gfp::par-2* and *pkc-3(ne4246); gfp::par-2* embryos treated either with *ctrl(RNAi)* or *gsp-2(RNAi)* (RNAi by feeding). Acquisition of midplane fluorescent images begins during the early establishment phase, and frames are captured every 10 s. In *gfp::par-2* embryos (both *ctrl(RNAi)* and *gsp-2(RNAi)*) PAR-2 localizes at the posterior cortex in the one-cell stage embryo and in P1 in the two-cell stage embryo (n = 11 and n = 13, respectively). In *pkc-3(ne4246); gfp::par-2, ctrl(RNAi)* PAR-2 is uniformly distributed at the cortex in both one and two cell stage embryo (n = 15). After depletion of *GSP-2, pkc-3(ne4246); gfp::par-2* embryos showed PAR-2 posterior localization in one-cell stage embryo and in the P1 cell (n = 18). N = 4. n = number of embryos analyzed; N = number of independent experiments. Anterior is to the left and posterior to the right. Scale bar: 5 µm.

**Video 2.** Depletion of GSP-2 restores the cell cycle asynchrony between AB and P1 in *pkc-3(ne4246) embryos*. Control and *pkc-3(ne4246)* embryos after *ctrl(RNAi)* and *gsp-2(RNAi)* (RNAi by feeding). Acquisition of midplane DIC images from NEBD in P0 to four-cell stage. Frames are captured every 10 s. *gsp-2(RNAi)* in *pkc-3(ne4246) embryos restores AB and P1 asynchrony. These four examples were chosen because they had cell cycle time close to the mean of each population. *ctrl(RNAi)* n = 12, *gsp-2(RNAi)* n = 10, *pkc-3(ne4246); ctrl(RNAi)* n = 10, and *pkc-3(ne4246); gsp-2(RNAi)* n = 18. N = 3. n = number of embryos analyzed; N = number of independent experiments. Anterior is to the left and posterior to the right. Scale bar: 5 µm.

**Video 3.** *gsp-1(RNAi)* does not rescue PAR-2 posterior cortical localization in the *pkc-3(ne4246); gfp::par-2* embryos. *gfp::par-2* embryos treated either with *ctrl(RNAi)* or *gsp-1(RNAi)* (RNAi by feeding). Acquisition of midplane fluorescent images begins during the early establishment phase, and frames are captured every 10 s. In *gfp::par-2* embryos (both *ctrl(RNAi)* and *gsp-2(RNAi)*, n = 8) PAR-2 localizes at the posterior cortex in the one-cell stage embryo and in P1 in the two-cell stage embryo. In *pkc-3(ne4246); gfp::par-2, ctrl(RNAi)* and *gsp-1(RNAi)* PAR-2 is uniformly distributed at the cortex in both one- and two-cell stage embryo (n = 10 and n = 13, respectively). N = 3. n = number of embryos analyzed; N = number of independent experiments. Anterior is to the left and posterior to the right. Scale bar: 5 µm.

**Video 4.** GSP-1 and GSP-2 codepletion impairs PAR-2 posterior cortical localization. *gfp::par-2* embryos treated with *ctrl(RNAi), n = 10, ctrl(RNAi); gsp-1(RNAi), n = 11, and gsp-1/2(RNAi) class I, n = 11 and class II, n = 3 (RNAi by injection). N = 3. Acquisition of midplane fluorescent images begins during the early establishment phase, and frames are captured every 10 s. In *gfp::par-2* embryos (both *ctrl(RNAi)* and *gsp-2(RNAi)*, n = 8) PAR-2 localizes at the posterior cortex in the one-cell stage embryo and in P1 in the two-cell stage embryo. In *gfp::par-2 gsp-1/2(RNAi) class I embryos, PAR-2 is detected in the cytoplasm, whereas in *gsp-1/2(RNAi) class II embryos, PAR-2 is weakly detected at the cortex compared to the *ctrl(RNAi)*. N = 3. n = number of embryos analyzed; N = number of independent experiments. Anterior is to the left and posterior to the right. Scale bar: 5 µm.

**Video 5.** The PP1 motif in PAR-2 is required for its localization at the posterior cortex. *gfp::par-2°* (the symbol ° indicates a mixture between wild-type *gfp::par-2* homozygous worms expressing wild-type *gfp::par-2* and heterozygous worms expressing wild-type *gfp::par-2* from the other allele) and *gfp::par-2(RAFA)* homozygote mutant embryos. Acquisition of midplane fluorescent images begins during the early establishment phase, and frames are captured every 10 s. In *gfp::par-2°* embryos PAR-2 localizes at the posterior cortex in the one-cell stage embryo and in P1 in the two-cell stage embryo (n = 16). In *gfp::par-2(RAFA)* embryos, PAR-2 is mostly detected in the cytoplasm (n = 17). N = 6. n = number of embryos analyzed; N = number of independent experiments. Anterior is to the left and posterior to the right. Scale bar: 5 µm.

**Video 6.** Two-cell stage *gfp::par-2(RAFA)* embryos divide synchronously. Acquisition of midplane DIC images from NEBD in P0 to four-cell stage for *gfp::par-2°* and *gfp::par-2(RAFA) n = 14 and n = 18, respectively). Frames are captured every 10 s. In *gfp::par-2(RAFA) embryos, AB and P1 divide synchronously. These four examples were chosen because they had cell cycle time similar to the mean of each population. N = 6. n = number of embryos analyzed; N = number of independent experiments. Anterior is to the left and posterior to the right. Scale bar: 5 µm.

**Video 7.** *gfp::par-2(L165V)* mutant embryos show aberrant PAR-2 cortical localization. *gfp::par-2* and *gfp::par-2(L165V)* embryos. Acquisition of midplane fluorescent images begins during the early stage, and frames are captured every 10 s. In *gfp::par-2(L165V) embryos PAR-2 localizes at both the anterior and posterior cortex in the early phase and is removed from the anterior in most of the embryos during the first cell division compared to *gfp::par-2*, where PAR-2 show a normal localization at the posterior cortex (n = 25 and n = 14, respectively). N = 4. n = number of embryos analyzed. N = number of independent experiments. Anterior is to the left and posterior to the right. Scale bar: 5 µm.
Provided online are nine tables. Table S1 shows the genotypes of strains used in this study. Tables S2, S3, and S4 show all the reagents used for CRISPR. Table S5 shows the clones used for RNA interference. Tables S6 and S7 show the plasmids used for the two-hybrid experiment. Tables S8 and S9 show the statistical analyses used in this study.