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aPKC Cycles between Functionally Distinct PAR Protein Assemblies to Drive Cell Polarity

Graphical Abstract

Highlights

- Distinct aPKC-containing assemblies respond to cues and generate polarity signals
- Clustering of aPAR proteins on the membrane enables segregation by cortical flow
- PAR-3 promotes both loading and polarization of aPKC but limits its activity in vivo
- aPKC activity links cue-sensing and effector assemblies to drive efficient polarization

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In Brief

PAR polarity pathway-mediated cell polarization relies on a conserved network of proteins including PAR-3, CDC-42, PAR-6, and aPKC. Rodriguez, Peglion et al. uncover a division of labor whereby PAR-6 and aPKC cycle between distinct cue-sensing and effector assemblies that act cooperatively to polarize the one-cell C. elegans zygote.

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aPKC Cycles between Functionally Distinct PAR Protein Assemblies to Drive Cell Polarity

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SUMMARY

The conserved polarity effector proteins PAR-3, PAR-6, CDC-42, and atypical protein kinase C (aPKC) form a core unit of the PAR protein network, which plays a central role in polarizing a broad range of animal cell types. To functionally polarize cells, these proteins must activate aPKC within a spatially defined membrane domain on one side of the cell in response to symmetry-breaking cues. Using the Caenorhabditis elegans zygote as a model, we find that the localization and activation of aPKC involve distinct, specialized aPKC-containing assemblies: a PAR-3-dependent assembly that responds to polarity cues and promotes efficient segregation of aPKC toward the anterior but holds aPKC in an inactive state, and a CDC-42-dependent assembly in which aPKC is active but poorly segregated. Cycling of aPKC between these distinct functional assemblies, which appears to depend on aPKC activity, effectively links cue-sensing and effector roles within the PAR network to ensure robust establishment of polarity.

INTRODUCTION

A crucial step in the polarization of metazoan cells is the localization of conserved sets of polarity effectors, known as the partitioning-defective or PAR proteins, to discrete membrane-associated cortical domains. Regulation of PAR protein distribution is essential for the localized activation of signaling pathways that coordinate many aspects of embryonic development, including asymmetric cell division, epithelial organization, and embryo axis establishment (Goldstein and Macara, 2007; St Johnston and Ahringer, 2010). Although the precise details vary between systems, in most cases the conserved PDZ domain proteins PAR-3 and PAR-6, the small guanosine triphosphatase (GTPase) CDC-42 and atypical protein kinase C (aPKC) act together to establish polarity on one side of the cell and drive asymmetry of a range of downstream pathways (reviewed in Goehring, 2014; McCaffrey and Macara, 2012; Suzuki et al., 2004; Zlomek et al., 1982).

In Caenorhabditis elegans, PAR-3, PAR-6, CDC-42, and the aPKC ortholog, PKC-3, play an essential role in polarizing the one-cell embryo or zygote by defining an anterior domain and hence are referred to as anterior PARs or aPARs (Figures 1A–1C). An opposing set of posterior PARs or pPARs, consisting of PAR-1, PAR-2, LGL-1, and the CDC-42 GAP, CHIN-1, form a complementary domain at the posterior. Together, aPARs and pPARs define the anterior-posterior axis of the zygote and orchestrate an asymmetric division that restricts germline determinants to the posterior daughter cell (Beatty et al., 2010; Boyd et al., 1996; Etemad-Moghadam et al., 1995; Gotta et al., 2001; Guo and Kemphues, 1995; Hoege et al., 2010; Kay and Hunter, 2001; Kumfer et al., 2010; Tabuse et al., 1998; Watts et al., 1996).

Polarization is triggered by the sperm-donated centrosome via two semi-redundant pathways (Figures 1A and 1B). First, the centrosome induces actomyosin cortical flow away from the newly defined posterior pole, which transports membrane-associated aPAR proteins into the anterior (Cheeks et al., 2004; Goehring et al., 2011b; Mayer et al., 2010; Munro et al., 2004). Second, centrosomal microtubules promote local loading of PAR-2 in the posterior. PAR-2 then recruits PAR-1, which drives posterior exclusion of aPARs through phosphorylation of PAR-3 (Boyd et al., 1996; Hao et al., 2006; Motegi et al., 2011). Following this “establishment phase,” the zygote enters a “maintenance phase” during which mutual antagonism between anterior and posterior PARs ensures their continued asymmetric localizations (Boyd et al., 1996; Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Tabuse et al., 1998; Watts et al., 1996).

Anterior PAR protein function is mediated through the kinase activity of PKC-3, which can phosphorylate PAR-1, PAR-2, and LGL-1 and drive their dissociation from the membrane (Beatty et al., 2010; Hao et al., 2006; Hoege et al., 2010; Hurov et al., 2004; Motegi et al., 2011). PAR-3, PAR-6, and CDC-42 are all required for proper PKC-3 membrane localization (Gotta et al., 2004; Zlomek et al., 1982).
Figure 1. PKC-3 Kinase Inhibition Leads to Symmetric Division and Loss of Asymmetry of Downstream Polarity Markers

(A–C) Model for symmetry breaking by the PAR system in C. elegans. aPARs (red) initially occupy the membrane and pPARs (blue) are cytoplasmic (A, Meiosis II). A cue (purple) from the centrosome pair (black spheres) segregates aPARs into the anterior and promotes formation of a posterior PAR domain at the opposite pole (A, Establishment). PAR domains are then stable until cytokinesis (A, Maintenance) and drive polarization of cytoplasmic factors such as MEX-5/6 (green) and P granules (orange), which ensure the daughter cells acquire distinct fates (A, Two-cell). (B) Symmetry breaking can occur in two ways: (i) segregation of aPARs by cortical actomyosin flow (advection); and (ii) posterior PAR-2 loading. (C) A complex network of physical and regulatory interactions links the PAR proteins. Membrane binding (gray lines), physical interactions (black lines), as well as positive (→) and negative (⊥) feedback, are shown. Where links are indirect or unknown, dashed lines are used. Both CDC-42 and PAR-3 are required for stable membrane association of PAR-6/PKC-3. PAR-6 and PKC-3 depend on each other for membrane association. PAR-2, LGL-1, and presumably CHIN-1, are able to load onto the membrane independently. PAR-1 also binds membrane but requires PAR-2 to reach maximal concentrations. PKC-3 phosphorylates PAR-1, PAR-2, and LGL-1 and displaces them from the membrane. Exclusion of CHIN-1 from the anterior is dependent on PKC-3, but whether it is a direct target of PKC-3 is unknown. Together, PAR-1, via phosphorylation of PAR-3, and CHIN-1, by suppressing activated CDC-42, prevent invasion of the posterior domain by aPARs. PAR-3 and PAR-2 have been proposed to undergo oligomerization, which is thought to enhance their membrane association (noted by circular arrows). See recent reviews (Goehring, 2014; Hoege and Hyman, 2013; Motegi and Seydoux, 2013) for more information.

(D) Midsection confocal images of fixed zygotes stained for PAR-2 at polarity maintenance and two-cell stage comparing wild-type, pkc-3(ts), and pkc-3(RNAi) conditions.

(E) Midsection fluorescent images of mCherry:PAR-2-expressing zygotes at maintenance and two-cell stage in DMSO (control), CRT90-treated, and pkc-3(RNAi).
RESULTS

Acute Inhibition of PKC-3 Function Leads to Loss of Asymmetric Division

We took two approaches to inhibit PKC-3 kinase activity. First, we examined a previously identified temperature-sensitive allele of pkc-3, ne4246 (Fievet et al., 2012), which alters a conserved Asp residue (D386V) close to the active site. Strains carrying pkc-3(ne4246) are subsequently referred to as pkc-3(ts). Consistent with loss of PKC-3 function, in pkc-3(ts) zygotes at the restrictive temperature (25°C), PAR-2 is not restricted to the posterior membrane and is partitioned symmetrically into the two blastomeres at the first cell division (Figure 1D). Loss of asymmetry was quantified by the asymmetry index (ASI) (see STAR Methods), which measures the asymmetry of a feature, e.g., PAR-2 membrane intensity, relative to wild-type on a scale from zero (no asymmetry) to 1 (normal asymmetry) (Figure 1D and Movie S1). Results below and in Figure S1 indicate that loss of asymmetry in pkc-3(ts) zygotes is due to loss of PKC-3 activity rather than degradation.

In parallel, we examined PKC-3 inhibitors in permeable, perm-1(RNAi) embryos (Carvalho et al., 2011) to identify compounds that yielded a PKC-3 deficient polarity phenotype. One compound, CRT0103390 (CRT90), a derivative of CRT0066854 (Figures S2A–S2C) (Kjær et al., 2013; Dorsey et al., 2013) resulted in embryos that progressed normally through the cell cycle but showed loss of PAR-2 asymmetry and divided symmetrically (Figure 1E and Movie S1). CRT90 embryos exhibited other common phenotypes associated with pkc-3(RNAi) and pkc-3(ts), including simultaneous division of the two daughter cells and ectopic spindle rotation in the anterior daughter cell, leading to a chain-like arrangement of cells in the 4-cell embryo (data not shown). As expected for an inhibitor of PKC-3 activity, CRT90 treatment caused loss of asymmetry in other posterior PAR proteins (PAR-1, LGL-1, and CHIN-1) as well as the loss of cytoplasmic asymmetry in the cell fate determinants PIE-1 and MEX-5 (Figure 1F).

PKC-3 Inhibition Yields Distinct Phenotypes from PKC-3 Depletion

We next investigated the distributions of the anterior PAR proteins when PKC-3 activity was inhibited, and compared these to those observed when PKC-3 was depleted by RNAi. Normally, PAR-3, PAR-6, and PKC-3 are efficiently segregated into the anterior membrane and is partitioned symmetrically into the two blastomeres at the first cell division (Figure 1D). Loss of asymmetry was quantified by the asymmetry index (ASI) (see STAR Methods), which measures the asymmetry of a feature, e.g., PAR-2 membrane intensity, relative to wild-type on a scale from zero (no asymmetry) to 1 (normal asymmetry) (Figure 1D and Movie S1). Results below and in Figure S1 indicate that loss of asymmetry in pkc-3(ts) zygotes is due to loss of PKC-3 activity rather than degradation.

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Figure 2. Membrane Localization of PAR-6/PKC-3 Is Decoupled from PAR-3 when PKC-3 Is Inactive

(A–F) Representative midsection confocal images of live and fixed zygotes at establishment and maintenance phase comparing control (DMSO, wild-type), *pkc-3*(RNAi), and PKC-3-inhibited (CRT90, *pkc-3*(ts)) conditions. PAR-6 (A and B) and PKC-3 (C and D) show loss of asymmetric membrane staining in PKC-3-inhibited zygotes at both establishment and maintenance phase (posterior localization indicated by white arrowheads). In *pkc-3*(RNAi), PAR-6 is absent from the membrane at all times. PAR-3 (E and F) still polarizes in PKC-3-inhibited zygotes, but becomes weaker and less asymmetric during maintenance phase. Note that (B), (D), and (F) show the same wild-type and TS zygotes with the PAR-3 boundary position in TS indicated (red arrowheads) to allow comparison: PAR-6 and PKC-3 are clearly visible at the posterior membrane (white arrowheads), while PAR-3 is undetectable, as in wild-type. Bright foci in (D) are non-specific centrosome staining.

(G and H) Normalized ASI measurements for late establishment phase datasets represented in (A) to (F). ASI is normalized to control (wild-type [WT] or DMSO) for each protein.

(I and J) Anterior to posterior membrane distributions of PAR-3 (red) and PKC-3 (black) in wild-type (I) and *pkc-3*(ts) (J) embryos. Arrows highlight the posterior extension of PKC-3 relative to PAR-3. Mean ± SD is shown.

(legend continued on next page)
Kemphues, 1999). By contrast, PAR-3 remains membrane associated and segregates into the anterior, but this population is generally reduced compared to wild-type and is lost as the cell proceeds through mitosis (Figure 2E) (Tabuse et al., 1998).

Unlike pck-3(RNAi), when PKC-3 is inhibited by the D386V mutation or CRT90, PAR-6 and PKC-3 remain membrane associated, fail to segregate efficiently to the anterior, and a significant pool of both proteins remains localized at the posterior pole resulting in a loss of asymmetry relative to controls (Figures 2A–2D, 2G, and 2H; Movie S2). In contrast to PAR-6 and PKC-3, PAR-3 still segregates into the anterior during the establishment phase in PKC-3-inhibited zygotes (Figures 2E and 2F, Establishment). The domain is typically somewhat enlarged relative to wild-type zygotes, but PAR-3 asymmetry remains high and PAR-3 is absent from the posterior pole (Figures 2G and 2H). Following the establishment phase, PAR-3 levels at the membrane decline and become more symmetric (Figures 2E and 2F, Maintenance).

The distinct response of PAR-3 versus PAR-6 and PKC-3 is particularly clear in the quantification of dual-labeled fixed zygotes. In wild-type zygotes, the boundaries of the PAR-3 and PKC-3 domains are positioned similarly at the center of the zygote, although the PKC-3 domain extends a few microns further into the posterior (Figures 2I and 2K, WT). By contrast, in PKC-3-inhibited embryos, PKC-3 extends significantly further into the posterior compared with PAR-3, a condition we refer to as “decoupled” (Figures 2J–2L, TS). Consistent with this decoupling, upon PKC-3-inhibition we observe a decrease in co-localization between PAR-3 and PAR-6 at the membrane/cortex, even in the anterior domain where these PAR proteins overlap (Figure S3). Thus, we conclude that PKC-3 is required for the normally tight coupling between PAR-6/PKC-3 and PAR-3 during symmetry breaking.

These results point to an unappreciated complexity in the assembly and regulation of the PAR proteins at the cell membrane:

First, the loss of membrane-associated PAR-6 in PKC-3-depleted zygotes, but not in PKC-3-inhibited zygotes (Figure 2A), shows that disruption of PKC-3 activity or the resulting invasion of pPARs into the anterior (Figures 1D–1F) do not account for loss of PAR-6 membrane association. Rather, there appears to be a requirement for PKC-3 protein itself to target and stabilize PAR-6 at the membrane. Consistent with this interpretation, mutations in par-2 and par-1 fail to rescue PAR-6 membrane localization in pck-3(RNAi) zygotes (Figure S4).

Second, in PKC-3-inhibited zygotes, anterior and posterior PAR protein distributions on the membrane overlap (Figure 2M). Posterior PAR proteins are thought to directly antagonize the ability of anterior PAR proteins to associate with the membrane, yet in these zygotes aPARs appear resistant to pPAR antagonism. PKC-3 inhibition could conceivably affect the activity of posterior PARs. However, we found that PAR-1 kinase activity, as measured by MEX-5 mobility (Griffin et al., 2011), appears normal in PKC-3-inhibited zygotes (Figures S2D–S2F). Thus, PKC-3 activity appears necessary to render anterior PARs sensitive to the antagonistic effects of posterior PARs, challenging the simple paradigm of mutual antagonism, which would predict pPAR dominance.

Finally, decoupling of PAR-6/PKC-3 from PAR-3 localization in PKC-3-inhibited zygotes during symmetry breaking suggests that PKC-3 drives formation of distinct PAR complexes or assemblies during polarity establishment in the C. elegans zygote. Contrary to what has been observed in Drosophila epithelia, where aPKC activity promotes decoupling of PAR-3 from PAR-6/aPKC and their targeting to distinct sites (Morais-De-Sa et al., 2010), here we observe the opposite: PKC-3 kinase activity is implicated in coupling the behaviors of PAR-3 and PAR-6/PKC-3, allowing their coordinated segregation during symmetry breaking.

PKC-3 Inhibition Promotes PAR-3-Independent Formation of CDC-42-Dependent PAR-6/PKC-3 Assemblies

If PKC-3 inhibition favors formation or trapping of a distinct functional assembly, we reasoned that it might affect the normal dependencies of PAR-6 and PKC-3 on PAR-3 and CDC-42. PKC-3 and PAR-6 normally require both PAR-3 and CDC-42 to localize stably to the membrane (Beers and Kemphues, 2006; Sailer et al., 2015). The dependency on PAR-3 is stronger: PKC-3 and PAR-6 fail to localize to the membrane in PAR-3-depleted zygotes (par-3(RNAi) in Figures 3A–3F) (Tabuse et al., 1998). By contrast, in CDC-42-depleted zygotes, PKC-3 and PAR-6 initially localize to the membrane and segregate to the anterior, but their membrane localization is gradually lost during the maintenance phase, becoming weaker and more uniform as zygotes approach cytokinesis (cdc-42(RNAi) Figures 3A–3F and Movie S4) (Beers and Kemphues, 2006; Gotta et al., 2001; Motegi and Sugimoto, 2006; Sailer et al., 2015; Schonegg and Hyman, 2006). Importantly, depletion of PAR-1 or PAR-2, which invade the anterior in the absence of PAR-3 or CDC-42 (Etemad-Moghadam et al., 1995; Gotta et al., 2001; Kay and Hunter, 2001; Schonegg and Hyman, 2006), fails to rescue PAR-6 membrane localization in these conditions (Figure S4). Thus, both PAR-3 and CDC-42 are directly required to promote membrane association of PAR-6 and PKC-3.

We find that under conditions of PKC-3 inhibition (D386V or CRT90), PKC-3 and PAR-6 no longer depend on PAR-3 to localize to the membrane (Figures 3A–3F and S5A; Movies S3 and S4). The degree of localization varies between the two methods of PKC-3 inhibition, possibly reflecting differences in the mechanism or timing/kinetics of kinase inhibition. By contrast, CDC-42 is still required for PKC-3 and PAR-6 membrane localization in PKC-3-inhibited zygotes (Figures 3A–3F, S5A, and SSB; Movie S4), indicating that CDC-42 is still required for PAR-6/PKC-3 membrane targeting even when PKC-3 is inhibited. Consistent with previous work showing that
Figure 3. PKC-3 Inhibition Promotes PAR-3-Independent Formation of CDC-42-Dependent PAR-6/PKC-3 Assemblies

(A–C) Representative midsection confocal images of live embryos at maintenance phase showing GFP::PKC-3 (A) or PAR-6::GFP (B and C) of DMSO, CRT90-treated, and pkc-3(ts) zygotes subject to RNAi as indicated.

(D) Quantification of rescue for datasets represented in (A) to (C), normalized to membrane signal in control RNAi and CRT90-treated/pkc-3(ts) zygotes for each dataset.

(E) Representative midsection confocal images of wild-type and pkc-3(ts) zygotes during polarity establishment subject to RNAi as indicated and immunostained for PKC-3.

(F) Quantification of rescue as measured by anterior domain cortical intensity of PKC-3 for datasets represented in (E). For each zygote, anterior PKC-3 cortical intensity is divided by cytoplasmic intensity. Values greater than 1 indicate presence at the membrane. Mean ± 95% confidence interval (CI) (N) is shown. See STAR Methods for further details.

(G) Representative midsection confocal images during polarity establishment of wild-type and pkc-3(ts) embryos upon cgef-1(RNAi), stained for PKC-3. Scatterplot representing the anterior domain cortical intensity of PKC-3 as in (F) in cgef-1(RNAi) and pkc-3(ts);cgef-1(RNAi). Mean ± 95% CI (N) is shown. *p < 0.01, ****p < 0.0001, ns, not significant. Scale bars, 10 μm. See also Figure S5; Movies S3 and S4.
Figure 4. Segregation of Anterior PAR Proteins Involves Cortical Clusters

(A) Representative cortical images of PAR-3, PKC-3, CDC-42, and PH-PLCΔ1 in late-establishment and maintenance-phase zygotes along with zoom of inset region (yellow box).

(B) Time-averaged cortical images spanning 180 s reveal anterior-directed tracks of cortical clusters of PAR-3, PAR-6, and PKC-3. Insets highlight the motion (arrows) of a representative single cluster in the image above.

(C) Cluster index for the full dataset in (A) and PAR-6::GFP (images not shown). Significance between establishment and maintenance: p < 0.01 for PAR-3, PAR-6, and PKC-3.

(D) Representative midsection or cortical images of PAR-3::GFP (WT) or PAR-3ΔCR1::GFP (ΔCR1) with and without co-expression of the membrane tether PH::GBP (+GBP) shown at symmetry-breaking, establishment, or maintenance phase. Note enhancement of membrane signal visible in GBP-expressing zygotes viewed in midsection and lack of bright clusters of PAR-3ΔCR1::GFP viewed at the cortex.

(E) Cluster index for WT and ΔCR1 in the presence of the PH-GBP membrane tether (+GBP) along with magnification of insets (yellow dashed-line rectangles in D) indicated to highlight the difference in clustering.

(F) Representative midplane images of WT and ΔCR1 subject to GBP-membrane targeting showing defective segregation of ΔCR1. Images shown are from late-establishment phase.

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PAR-6/aPKC are typically associated with an active, guanosine triphosphate (GTP)-bound form of CDC-42 (Atwood et al., 2007; Gotta et al., 2001; Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2000), we found that decreasing CDC-42/GTP by depletion of the CDC-42 GEF, CGEF-1, reduces membrane association of PAR-6/PKC-3 in pkc-3(ts) embryos compared with wild-type, while leaving PAR-3 levels unchanged (Figures 3G and SSA–SSE). Finally, PKC-3 remains dependent on PAR-6 in PKC-3-inhibited embryos, consistent with PAR-6 being required to mediate the interactions of the PAR-6/PKC-3 heterodimer with PAR-3 and/or CDC-42 (Figures 3A and 3D–3F; Movie S4) (Joberty et al., 2000; Qiu et al., 2000), as well as our general findings that PKC-3 and PAR-6 respond similarly in all assays described.

Thus, inhibition of PKC-3 activity allows PKC-3 to bypass its normal requirement on PAR-3 to load onto the membrane and form stable membrane-associated CDC-42/GTP-dependent complexes. We postulate that it is this dependency on PAR-3, enforced by PKC-3 kinase activity, that ensures the coupled distributions of PKC-3 and PAR-3 in the embryo.

**Segregation of Anterior PAR Proteins Is Associated with PAR-3-Dependent Clustering at the Membrane**

So far, we have shown that inhibition of PKC-3 kinase activity promotes CDC-42-dependent assemblies, and in so doing prevents PKC-3 and PAR-6 from segregating efficiently with PAR-3 into the anterior during symmetry breaking. Previous work has shown that the efficient segregation of anterior PAR proteins is due to advective transport by anteriorly-directed actomyosin cortical flow (Cheeks et al., 2004; Goehring et al., 2011b; Munro et al., 2004). Because PAR-3 continues to be segregated efficiently in PKC-3-inhibited zygotes, we reasoned that PKC-3 inhibition may selectively alter the molecular organization of PAR-6 and PKC-3 at the membrane relative to PAR-3, which would be consistent with the observed shift toward CDC-42-dependent PKC-3 assemblies.

To investigate these possibilities, we imaged PAR-3, PAR-6, and PKC-3 at the membrane using variable-angle epifluorescence microscopy (VAEM or pseudo-TIRF) (Konopka and Bednarek, 2008). All three proteins exhibit a distinct clustered appearance during the polarity establishment phase, consistent with reports of non-homogenous distributions of PAR proteins at the membrane (Figures 4A and 5A, Establishment) (Munro et al., 2004; Robin et al., 2014; Sailer et al., 2015). Similar to previous analysis of PAR-6 (Munro et al., 2004), we find that clusters of PAR-6, PAR-3, and PKC-3 move in a highly directional manner in the direction of cortical flow, coinciding with increasing overall asymmetry (Figure 4B and Movie SS). While aPAR clusters have been noted (Hung and Kemphues, 1999; Munro et al., 2004; Sailer et al., 2015), the relationship between clustered and non-clustered PAR proteins and their ability to segregate in response to flow has not been explored.

To test whether clustering is a key driver of aPAR segregation, we examined whether PAR-3 transport depends on its ability to cluster. PAR-3 contains a conserved CR1 oligomerization domain, which is required for membrane binding and is targeted by PAR-1 kinase to induce displacement form the membrane (Benton and St Johnston, 2003; Feng et al., 2007; Li et al., 2010; Mizuno et al., 2003). We reasoned that this domain would be required for clustering; however, because mutations in the CR1 domain disrupt membrane binding (Figure 4D, WT versus ΔCR1), assessing clustering and segregation of a PAR-3ΔCR1 mutant requires an alternative mode of membrane targeting. We restored membrane localization of GFP:PAR-3ΔCR1 using a membrane-tethered anti-GFP nanobody (PH-GBP) and compared this with the behavior of GFP:PAR-3 (wild-type) that was also tethered to the membrane via PH-GBP. Targeting both wild-type and ΔCR1 to the membrane with PH-GBP reduces potential confounding effects of PAR-1-induced membrane displacement in the posterior. Thus, differences in segregation in PAR-3ΔCR1 relative to wild-type should be due to changes in clustering rather than differential sensitivity to PAR-1.

Consistent with oligomerization being required for clustering, membrane-tethered PAR-3ΔCR1 exhibited more diffuse membrane localization compared with wild-type controls (Figures 4D and 4E, WT + GBP versus ΔCR1 + GBP). PAR-3ΔCR1 also showed reductions in both segregation into the anterior and overall asymmetry (Figures 4F–4H and Movie SS). Thus, the ability of the CR1 domain to drive formation of membrane-associated PAR-3 clusters ensures that PAR-3 is efficiently transported by cortical flows in addition to its known role in promoting membrane association.

**Balance between PAR-3 and CDC-42 Assemblies Tunes Cortical Organization and Sensitivity to Cortical Flow**

The correlation between lack of PAR-3 clustering and defects in advective transport prompted us to examine the organization of anterior PAR proteins at the membrane in more detail. Although PAR-6 and PKC-3 exhibit a distinct clustered appearance similar to PAR-3 during polarity establishment, clusters are less pronounced and accompanied by a background of a more diffuse population (Figures 4A, 4C, and 5A). By contrast, CDC-42 exhibits a more uniform signal overall that resembles typical membrane markers, such as the PIP2 (phosphatidylinositol-4,5-bisphosphate) probe PH-PLCδ1 (Figures 4A and 4C). Membrane markers do exhibit enriched signals in membrane folds and protrusions, which are also enriched in the anterior, but these signals are clearly distinguishable from clusters.

With the transition into maintenance phase, clusters of PAR-6 and PKC-3 become less prominent and a diffuse population dominates. This change coincides temporally with a decrease in the prominence of PAR-3 clusters and an overall reduction in PAR-3 membrane localization (Figures 4A and 4C) as well as an increase in anterior CDC-42 activity (Figures SSC–SSE) (Kumfer et al., 2010). We therefore speculated that the mix of diffuse and clustered PAR-6/PKC-3 observed during establishment phase may reflect the distinct CDC-42- and PAR-3-dependent assemblies that we describe above. Consequently,
Figure 5. Regulation of PKC-3/PAR-6 Cluster Association by PAR-3/CDC-42 Balance Tunes Responsiveness to Cortical Flows
(A) Representative cortical images of PAR-6::GFP at late-establishment and maintenance-phase embryos for indicated conditions, shown along with a zoom of inset region (white boxes).
(B) Cluster index measurements of full dataset reveal a gradient of cluster association across conditions. Note that clustering decreases when embryos enter maintenance phase, except for CRT90/par-3(RNAi) embryos, which show minimal clustering even in establishment phase.
(C) ASI measurements of midsection images taken at late-establishment phase for a similar set of embryos as in (A) and (B), but expressing GFP::PKC-3, show a similar trend.
(D) Profiles of membrane signal for zygotes in (C) showing average (solid line) and full range of data (shaded) reveal shift of the PKC-3 domain boundary (arrows) toward the anterior in cdc-42(RNAi) embryos and toward the posterior in CRT90-treated zygotes, resulting in significantly (p < 0.01) smaller and larger domain sizes, respectively.

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cell-cycle-dependent changes in the balance between assembly types could effectively tune the system to promote efficient transport of aPAR species during the polarity establishment phase.

To test this hypothesis, we altered the balance between CDC-42- and PAR-3-dependent assemblies and monitored the corresponding changes in (1) organization at the membrane, and (2) segregation efficiency. In general, we find a striking correlation between quantitative measures of cortical clustering and overall asymmetry.

Depletion of CDC-42 is known to increase co-localization of PAR-6 with PAR-3 during polarity establishment (Beers and Kemphues, 2006). We find that this also increases overall clustering of PAR-6 (Figures 5A and 5B). Examination of PKC-3 distributions clearly reveals enhanced segregation, with increased ASI (Figure 5C) and a visibly steeper domain boundary that is shifted toward the anterior (Figure 5D), resulting in a smaller anterior domain (*p < 0.01). Consistent with these data, we also observe a tighter coupling between the PAR-3 and PKC-3 domain boundaries in dual-labeled fixed zygotes (Figure 5K).

In contrast to CDC-42 depletion, inhibition of PKC-3 using CRT90, which favors CDC-42-dependent assemblies, shows reduced clustering of PAR-6, which could be reduced further by also depleting PAR-3 (Figures 5A and 5B). Under these conditions that favor CDC-42-dependent assemblies and reduced clustering, PKC-3 segregated less efficiently than DMSO controls, exhibited a reduced ASI, and failed to be fully excluded from the posterior (Figures 5C and 5D). To confirm that this reduction in clustering and segregation is due to favoring CDC-42-dependent assemblies, we examined the effect of expressing CDC-42 (Q61L), which stabilizes the active GTP-bound form of CDC-42 (Aceto et al., 2006; Ziman et al., 1991). Unlike PKC-3 inhibition, CDC-42 (Q61L) does not efficiently bypass the normal dependence of PKC-3 on PAR-3 (Figures S5F–S5J). This suggests that inhibition of PKC-3 favors CDC-42-associated assemblies via a mechanism distinct from stabilizing the GTP-bound form of CDC-42. However, similar to what we see when PKC-3 is inhibited, expression of CDC-42 (Q61L) reduced clustering and resulted in less efficient segregation of PKC-3 (Figures 5F–5K). Thus, regardless of the mechanism by which we alter the balance between PAR-3- and CDC-42-dependent assemblies, we achieve similar effects on clustering and segregation. This is particularly striking when we plot mean cluster index versus asymmetry across all conditions at establishment phase (Figure 5E).

Because PAR proteins are known to regulate actomyosin dynamics (Cheeks et al., 2004; Munro et al., 2004), and changes in flow velocities could, in principle, affect advective transport (Goehring et al., 2011b), we wanted to confirm that clustering rather than potential changes in flow velocity were the cause of reduced segregation efficiency. Measurements of flow rates from yolk granule motion in differential interference contrast (DIC) images allowed us to test the relationship between cortical flow rates and asymmetry in individual zygotes. Consistent with anterior PARPs promoting their own segregation via stimulation of cortical flows, we find that PKC-3 inhibition results in a reduction of flow rates from approximately 6–10 μm/min in controls to approximately 2–5 μm/min in CRT90-treated embryos (Figure 5L). To test whether alterations in flow velocities could account for the observed segregation defects, we performed a partial depletion of MLC-4 to generate embryos with flow velocities of a similar range as observed in PKC-3-inhibited embryos (Figure 5L). Despite a similar range of flow velocities, MLC-4-depleted zygotes show a minimal reduction in asymmetry versus controls (Figures 5M and S6A). Plotting flow velocity versus ASI reveals a weak decline in ASI as flow rates are reduced (Figure 5N). By contrast, CRT90-treated embryos show a lower ASI across the full range of observed flow rates (~2–5 μm/min, Figures 5N and S6A). Finally, to test whether restoring flows could rescue efficient segregation of PKC-3, we used RNAi to target the RhoGAPs, RGA-3/4, which results in excess actomyosin contractility and increased cortical flow rates (Fievet et al., 2012; Schonegg et al., 2007). Despite fully rescuing the moderate reduction in asymmetry of PAR-3 observed in mck-3(ts) embryos to levels indistinguishable from wild-type, RGA-3/4 depletion failed to restore asymmetry of PKC-3 (Figures S6B and S6C).

Together these data suggest that it is the failure of PKC-3 to associate with clusters rather than changes in flow rates that are the dominant factor in the decoupling between the localization of PAR-3 and PAR-6/PKC-3 observed in PKC-3-inhibited embryos. In fact, the resilience of PKC-3 asymmetry in embryos partially depleted of MLC-4 suggests that there is a relatively low threshold velocity required for efficient segregation of aPAR proteins by cortical flow, provided aPARs are able to associate normally into clusters.

We therefore conclude that although both PAR-3 and CDC-42 are critical for normal PAR-6/PKC-3 localization at the membrane in wild-type embryos, they drive formation of distinct aPAR assemblies, with distinct physical properties and...
responsiveness to cortical flow: PAR-3-dependent assemblies exhibit pronounced clustering, at least during the establishment phase, and are efficiently segregated by cortical flow. By contrast, CDC-42-dependent assemblies are more diffuse, likely reflecting enhanced diffusional mobility, and are inefficiently segregated by flow. Importantly, the balance between these two species appears to be subject to cell-cycle-dependent regulation to ensure maximal clustering and transport during the period of peak actomyosin cortical flows.

A PKC-3 Membrane-Targeting Assay Reveals Opposing Roles for PAR-3 and CDC-42 in Regulating PKC-3 Activity

We next sought to explore whether there were other functional differences in these two types of assemblies. Specifically, we wondered whether PAR-3 and CDC-42 may have distinct regulatory effects on PKC-3 activity in vivo, which is difficult to analyze given their roles in PKC-3 membrane loading. While CDC-42 is generally thought to play an activating role (Atwood et al., 2007; Gotta et al., 2001; Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2000), the roles for PAR-3 and PAR-6 are less clear and may vary in different contexts (Achilleos et al., 2010; Atwood et al., 2007; David et al., 2013; Graybill et al., 2012; Lin et al., 2000; McCaffrey and Macara, 2009; Wirtz-Peitz et al., 2008).

To directly assess whether PKC-3 activity differs in PAR-3-associated and CDC-42-associated assemblies in vivo, we targeted PKC-3 to the membrane by fusing it to the C1B domain of human PKCα, which can be induced to bind the membrane by the addition of phorbol ester (Figure 6A) (Lekomtsev et al., 2012). This bypasses the membrane-binding requirement of PKC-3 on PAR-3, PAR-6, and CDC-42, allowing us to test their contribution to PKC-3 activity by monitoring membrane removal of the PKC-3 target, PAR-2.

In the absence of phorbol ester, C1B-PKC-3 mirrors endogenous PKC-3 localization and is anteriorly enriched, with PAR-2 restricted to the posterior as in wild-type (Figure 6B, No PMA). Upon addition of 100 μM phorbol 12-myristate 13-acetate (PMA), C1B-PKC-3 is recruited uniformly to the membrane and the PAR-2 domain shrinks, consistent with an increase in posterior PKC-3 activity (Figure 6B, +PMA; Figure 6C; Movie S6). The reduction in PAR-2 domain size is not seen in the absence of PMA, when targeting the C1B domain alone to the membrane, or if we inhibit PKC-3 with CRT90 (Figures 6B and 6D). The failure to completely remove PAR-2 in polarized zygotes is not simply due to PAR-2 being concentrated in a domain, because ectopic PAR-2 domains that form in meiotic arrest mutants, e.g., mei-1 and emb-27 (Wallenfang and Seydoux, 2000), are rapidly cleared (Figure S7).

In par-3, par-6, or cdc-42(rnaI) zygotes, both endogenous PKC-3 and the C1B–PKC-3 fusion are cytoplasmic in the absence of PMA, allowing PAR-2 to localize uniformly to the membrane (Figures 6E and 6F). In par-6 and cdc-42(rnaI) zygotes, membrane targeting of C1B–PKC-3 (+PMA) has no effect on PAR-2 distribution: it remains uniformly enriched at the membrane with no difference compared with controls in which C1B alone is targeted to the membrane (Figures 6E and 6F; Movie S7). Thus, both PAR-6 and CDC-42 are required for PKC-3 activity in vivo.

By contrast, membrane targeting of C1B-PKC-3 in par-3(rnaI) zygotes induces rapid loss of PAR-2 from the membrane, with near complete removal within minutes (Figures 6E and 6F; Movie S7). The displacement of PAR-2 is stronger than in wild-type zygotes, suggesting that PAR-3 normally acts to inhibit or suppress PKC-3 activity (Figure 6B, +PMA). Thus PAR-3 has two roles in vivo: it promotes PKC-3 membrane targeting while at the same time limiting its activation, reconciling in vivo reports whereby PAR-3 positively regulates PAR polarity (Achilleos et al., 2010; McCaffrey and Macara, 2009) with data indicating that PAR-3 can inhibit PKC-3 activity in vitro (Graybill et al., 2012; Lin et al., 2000; Soriano et al., 2016).

DISCUSSION

Taken together, our data suggest that efficient polarization requires PKC-3 to cycle between functionally specialized modules of the anterior PAR network: a PAR-3-dependent module that segregates in response to symmetry-breaking signals, but which is inactive, and a CDC-42-dependent module that uses spatial information provided by PAR-3 to create an anterior domain of PKC-3 activity on the membrane (Figure 7).

In previous work, we showed that the diffusion and membrane dissociation rates of aPARs were in principle sufficient to explain segregation in response to flows (Goehring et al., 2011b). Here we show that segregation of aPARs is directly linked to PAR-3-dependent clustering. Clustering reduces the effective diffusion of membrane-associated aPARs, which should favor advective transport. Alternatively, the sheer size of clusters may allow them to sense flow that would not affect individual proteins, possibly by allowing them to extend into the cortical actomyosin layer. Regardless of the physical mechanism, as we show here, clustering drives robust segregation of PAR-3 by cortical flow. This fact, coupled with PAR-3 exclusion from the posterior by PAR-1-dependent phosphorylation (Motegi et al., 2011), supports a model in which PAR-3 is responsible for sensing asymmetry-generating cues. Importantly, once it is asymmetric, PAR-3 provides a landmark for polarized loading of PAR-6/PKC-3, explaining recent observations that PAR-6 loads preferentially in the anterior of polarized embryos (Sailer et al., 2015).

Because our in vivo PKC-3 activity assay indicates that PKC-3 activity is suppressed within PAR-3-dependent assemblies, PAR-6/PKC-3 molecules must be converted into an activated CDC-42-dependent species, a state that we show is non-clustered and diffusive. Whereas this diffusive behavior of CDC-42-assemblies is a disadvantage for transport by cortical flow, it is an advantage for creating a uniform, wider-range field of PKC-3 activity that can efficiently exclude pPARs. If the same complexes had to respond to flow and exclude pPARs, there would be a trade-off between efficiency of transport by flow and uniformity of pPAR inhibition at the anterior.

For this field of activated CDC-42-dependent PKC-3 assemblies to remain coupled to the spatial information provided by PAR-3, two conditions must be satisfied. First, PKC-3 membrane localization must be dependent on PAR-3, and second, diffusion of CDC-42-associated PKC-3 away from loading sites must be limited, with PKC-3 ultimately being released back into the cytoplasm, where it again becomes dependent on PAR-3. This turnover restricts the effective distance these
Figure 6. PAR-3 and CDC-42 Have Opposing Regulatory Roles in an In Vivo PKC-3 Activity Assay

(A) C1B targeting strategy for inducing PKC-3 membrane loading by PMA. PKC-3 kinase activity is monitored by following loss of PAR-2 from the membrane.

(B) Zygotes expressing GFP::C1B alone (GFP::C1B-Ø) or GFP::C1B-PKC-3 along with mCherry::PAR-2 were subject to the indicated treatment. Note that uniform membrane targeting of C1B-PKC-3 leads to reduction of PAR-2 domain size, whereas omitting PMA or expressing C1B alone has no effect. Right: cartoon representation of results.

(C) Quantification of PAR-2 domain size ratio for embryos shown in (B).

(D) PAR-2 retention in GFP::C1B-PKC-3 expressing zygotes treated with PMA and CRT90 confirms that induced PAR-2 loss is dependent on PKC-3 kinase activity.

(E) Zygotes expressing mCherry::PAR-2 with GFP::C1B-Ø or GFP::C1B-PKC-3 subject to par-6, cdc-42, or par-3[RNAi] before and 5 min after PMA addition.

(F) Quantification of PAR-2 cortex retention comparing GFP::C1B-PKC-3 and GFP::C1B-Ø zygotes after treatment with PMA as in (E).

Representative midsection confocal images are shown in (B), (D), and (E) before and 5 min after PMA/DMSO addition. ***p < 0.001. ns, not significant. Scale bars, 10 μm. See also Figure S7; Movies S6 and S7.
complexes can diffuse from their initial sites of formation, defining an effective “sphere of influence” around PAR-3 loading sites. Together, these requirements result in a cycle of localized membrane loading, activation, and release (Figure 7).

Our data suggest that the first of these requirements, PAR-3-dependent loading, is dependent on the kinase activity of PKC-3 itself, although the precise mechanism is unclear. Given the limited ability of CDC-42 (Q61L) to rescue PKC-3 membrane localization in PAR-3-depleted embryos, PKC-3 is likely to act at the step of CDC-42 complex generation, either inhibiting its own association with CDC-42, and/or destabilizing nascent CDC-42/PAR-6/PKC-3 assemblies. We speculate that it could be the very act of inhibiting PKC-3 through which PAR-3 promotes generation of stable CDC-42-dependent assemblies, but further work will be required to reveal the details of this molecular handover. Because the inhibitory role of PAR-3 appears to be broadly conserved (David et al., 2013; Graybill et al., 2012; Lin et al., 2000; McCaffrey et al., 2012; Soriano et al., 2016), this apparent paradoxical role of PAR-3 in promoting formation of membrane-associated aPKC complexes, yet also suppressing PKC-3 activity, may be a general feature of aPKC regulation.

How diffusion of CDC-42-associated PKC-3 is limited also remains unclear. Measurements elsewhere suggest that the distance these activated assemblies travel is on the order of 5–10 μm (Goehring et al., 2011a; Robin et al., 2014), consistent with the PKC-3 gradient extending further into the posterior than PAR-3 during the establishment phase even in wild-type embryos (see Figure 2I). Because CDC-42 assemblies appear to be resistant to removal by posterior PAR proteins (Figure 2M), including the CDC-42 GAP CHIN-1, it seems likely that it is not preferential removal of these complexes in the posterior by pPARs, but rather the intrinsic lifetime of CDC-42-dependent PKC-3 assemblies that limits their diffusion into the posterior. This is compatible with a model in which aPKC undergoes asymmetric membrane loading but symmetric dissociation (Robin et al., 2014).

By loading PKC-3 via what we presume is an inhibited PAR-3-associated state, which must then be activated, PKC-3 localization and activation are segregated into distinct modules of the PAR network, which can be regulated independently. This division of labor may be critical for PAR proteins, which operate across diverse contexts, where the polarity cues, substrates, scales, and even the concentrations of PAR molecules themselves may vary substantially. Even within the C. elegans zygote, the mechanisms of PAR segregation vary. During polarity establishment, when cortical flow is the major cue for anterior PAR segregation, PAR-3 clustering is prominent (Cheeks et al., 2004; Goehring et al., 2011b; Munro et al., 2004). As the system enters the maintenance phase, flow ceases and continued aPAR segregation becomes dependent on the activity of PAR-1 and CHIN-1 (Beatty et al., 2013; Guo and Kemphues, 1995; Kumfer et al., 2010; Sailer et al., 2015). Notably, clustering appears to be reduced during this phase, which Dickinson et al. (2017) show is dependent on PLK-1. This change in PAR molecular organization potentially reflects the shift in the spatial signals to which the PAR network must respond. At the same time, despite these changes, PKC-3 activity must remain efficient at displacing pPARs from the anterior domain, highlighting the adaptability of the PAR network.

In summary, here we have identified a critical role for the separation of signal-receiving and signal-transducing functions between modules of the aPAR network that are distinct, but
coupled via dynamic exchange of the shared signaling component, PKC-3. We suggest that functional specialization of coupled modules resolves potential molecular constraints between components that must be sensitive to polarity cues and those that must propagate the signals. It further allows the system to independently modulate responsiveness to cues as well as the extent and strength of the output signal. The adaptability of such a paradigm suggests it is likely to be a common strategy in patterning systems.

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and seven movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2017.07.007.

**AUTHOR CONTRIBUTIONS**


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**REFERENCES**


### STAR METHODS

#### KEY RESOURCES TABLE

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<td>C. elegans: NWG0003 [par-2(ts); gfp:: par-6]; def-7(e1372) par-2(ts) III; unc-119 (ed3) III; dds18 [gfp::par-6(cDNA); unc-119(+)]</td>
<td>this paper</td>
<td>NWG0003</td>
</tr>
<tr>
<td>C. elegans: NWG0012 [gfp::c1b]; unc-119(ed3) III; crkl4[Fpie-1::sgfp:: c1b::pie-1utr; unc-119(+)]</td>
<td>this paper</td>
<td>NWG0012</td>
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<td>C. elegans: NWG0016 [gfp::c1b::pkc-3]; unc-119(ed3) III; crkl4[Fpie-1::sgfp:: c1b::pkc-3::pie-1utr; unc-119(+)]</td>
<td>this paper</td>
<td>NWG0016</td>
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<td>C. elegans: NWG0021 [gfp::c1b::pkc-3]; MCherry::par-2; unc-119(ed3) III; dds131[pie-1::mCherry::par-2::pie-1utr; unc-119(+)]; crkl4[Fpie-1::sgfp:: c1b:: pkc-3::pie-1utr; unc-119(+)]</td>
<td>this paper</td>
<td>NWG0021</td>
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<td>C. elegans: NWG0022 [gfp::c1b::MCherry:: par-2; unc-119(ed3) III; dds131[pie-1::mCherry::par-2::pie-1utr; unc-119(+)]; crkl4[Fpie-1::sgfp:: c1b:: pie-1utr; unc-119(+)]</td>
<td>this paper</td>
<td>NWG0022</td>
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<tr>
<td>C. elegans: NWG0026 [par-6::gfp; MCherry:: par-2; par-6(1310)[par-6::gfp]]; unc-119 (ed3) III; dds131[pie-1::mCherry::par-2; unc-119(+)]</td>
<td>this paper</td>
<td>NWG0026</td>
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<td>C. elegans: NWG0027 [gfp::pkc-3; MCherry:: par-2; pkc-3(1309) [gfp::pkc-3]]; unc-119(ed3) III; dds131[pie-1::mCherry::par-2; unc-119(+)]</td>
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C. elegans

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<td>C. elegans: NWG0028[par-3::gfp::mCherry::par-6]; par-3[t298 [par-3::gfp]]; III; unc-119(ed3)[II]; ddIs26 [mCherry::T26E3.3::unc-199(+)]</td>
<td>this paper NWG0028</td>
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<td>C. elegans: NWG0039[par-1(ts); par-6::gfp]; par-1[p3310] V; par-6[t310][par-6::gfp] I</td>
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<td>C. elegans: NWG0047[PH::GBP]; unc-119[ed3] III; crkEx1[pNG19]; mex-5::PH[PLC1::V]; GBP::mKate::nmv-2UTR + unc-119(+) V; him-5(e1490) V</td>
<td>this paper NWG0047</td>
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<td>C. elegans: NWG0053[par-6::gfp::pkc-3[ts]]; par-6[t310][par-6::gfp] I; pkc-3[ne4246] II</td>
<td>this paper NWG0053</td>
</tr>
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<td>C. elegans: OD70[mCherry::PH::PLC1::V]; unc-119[ed3] III; llts44[pie-1p::mCherry::PH[PLC1::V] + unc-119(+) V</td>
<td>(Kachur et al., 2008)</td>
</tr>
<tr>
<td>C. elegans: SA131[gfp::cdc-42]; unc-119[ed3][II]; ts[pie-1::gfp::cdc-42; unc-119(+)]</td>
<td>(Moteigi and Sugimoto, 2006)</td>
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<td>C. elegans: TH129[gfp::par-2]; unc-119[ed3][II]; ddIs26[GBP::F58B6.3::unc-119(+)]</td>
<td>(Schonegg et al., 2007)</td>
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<td>C. elegans: TH159[mCherry::cdc-42]; unc-119[ed3][II]; ddIs48[WRM0625ba11] GLCherry::cdc-42; Cbr::unc-119(+)</td>
<td>Tony Hyman</td>
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<td>C. elegans: TH209[mCherry::par-2]; unc-119[ed3][II]; ddIs31[pie-1p::mCherry::par-2; unc-119(+)]</td>
<td>(Schonegg et al., 2007)</td>
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<td>C. elegans: TY3558[gfp::h::nis-11; b-tubulin::gfp]; rls[pie-1::GFPhis-11 III]; ojs1[1/2-tubulin::GFP]</td>
<td>CGC WB Strain: TY3558</td>
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<tr>
<td>C. elegans: UE37[pie-1::gfp::axEx73 [pie-1::gfp::rol-6[au1006] + N2 genomic DNA]; tubulin mCherry]</td>
<td>Carrie Cowan</td>
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<tr>
<td>C. elegans: WH423[mCherry::cdc-42[Q61L]]; Ppie-1[mcherry::cdc-42[Q61L]]</td>
<td>(Kumfer et al., 2010)</td>
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<td>C. elegans: WH497[gp::chin-1]; ojs69[pie-1::mGFPh::chin-1 + unc-119(+)]</td>
<td>CGC</td>
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<td>C. elegans: WH517[gp::wsp-1]; ojs40[pie-1::gfp::GBDwsp-1 + unc-119(+)]</td>
<td>CGC</td>
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<td>C. elegans: WM150[pie-3[ts]]; pkc-3[ne4246] II</td>
<td>(Fiavet et al., 2012)</td>
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<td>C. elegans: WS5018[gfp::cdc-42]; cdc-42[gk388]; ols295 [cdc-42p::gfp::cdc-42[genomic]; cdc-42 3'UTR + unc-119(+) II.</td>
<td>(Neukomm et al., 2014)</td>
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Oligonucleotides

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<tr>
<td>ptc-3[genomic] fwd: CCCACTAGTATGTCG</td>
<td>IDT DNA</td>
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<tr>
<td>TCTCCGACAT</td>
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<td>ptc-3[genomic] rev: CCCAGGCTTCCAGAC</td>
<td>IDT DNA</td>
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<td>TGAATCTCC</td>
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<td>PH-GBP gBlock: fwd: TCTCGTTTCTCATTGTATCTTCTC</td>
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<tr>
<td>PH-GBP gBlock: rev: ATGATGCCGCTTAGCTAGC</td>
<td>IDT DNA</td>
</tr>
<tr>
<td>Site-directed mutagenesis (PAM site) in pNG0018, fwd: GTCTGTTCGTAAGTCTGCTGATAACT</td>
<td>IDT DNA</td>
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</tbody>
</table>

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Contact for Reagent and Resource Sharing

Requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Josana Rodriguez (josana.rodriguez@ncl.ac.uk). CRT0103390 may be obtained through an MTA from Cancer Research Technology (jroffey@cancertechnology.com).

Experimental Model and Subject Details

C. elegans Strains and Maintenance

C. elegans strains were maintained on nematode growth media (NGM) under standard conditions (Brenner, 1974) at 16°C or 20°C unless otherwise indicated. Strains listed in the Key Resources Table. Note analysis of zygotes precludes determination of animal sex.

C. elegans Transgenic Animals

Following the scheme of (Lekomtsev et al., 2012), a codon-optimized (Redemann et al., 2011) sequence encoding the C1B domain from human PKCα (GenScript) was inserted into pTH699 via BamHI and Smal to generate a sfGFP::c1b fusion under control of the pie-1 promoter and -3′ UTR (pC1B-Ø). Genomic pkc-3 was amplified from fosmid WRM069dD11 (Source BioScience, WB Clone: WRM069dD11) using the following primers (fwd:cccactagtatgtcgtctccgacat; rev:cccaggccttcagactgaatcttcc) and inserted into pTH699 via BamHI and SmaI to generate a sfGFP::c1b fusion under control of the pie-1 promoter and -3′ UTR (pC1B-Ø).

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<tr>
<td>Site-directed mutagenesis (PAM site) in pNG0018, fwd: TGATATCGAAACAACACTG</td>
<td>IDT DNA</td>
<td>N/A</td>
</tr>
<tr>
<td>ctl (RNAi): fwd: ATCGATAAGCTTGTATCTCCTTG</td>
<td>IDT DNA</td>
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<tr>
<td>ctl (RNAi): rev: ACCGGCGGATCCCTAAATACGG</td>
<td>IDT DNA</td>
<td>N/A</td>
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Recombinant DNA

Fosmid: WRM069dD11 Source BioScience WB Clone: WRM069dD11
Plasmid: L4440 Addgene plasmid#1654
Plasmid: pUC57-C1B(codon-optimized) GenScript N/A
Plasmid: pTH699 Gift from Tony Hyman N/A
Plasmid: pC1B-Ø This paper N/A
Plasmid: pC1B-pkc-3 This paper N/A
Plasmid: CmKate2 MosSci vector Gift from Tony Hyman N/A
Plasmid: pNG0018 This paper N/A
Plasmid: pNG0019 This paper N/A
Ahringer Feeding RNAi: cdc-42 Source BioScience WB Clone: sjj_R07G3.1
Ahringer Feeding RNAi: emb-27 Source BioScience WB Clone: sjj_F10B5.6
Ahringer Feeding RNAi: par-3 Source BioScience WB Clone: sjj_F54E7.3
Ahringer Feeding RNAi: par-6 Source BioScience WB Clone: sjj_T26E3.3
Ahringer Feeding RNAi: pck-3 Source BioScience WB Clone: sjj_F09E5.1
Ahringer Feeding RNAi: perm-1 Source BioScience WB Clone: sjj_T01H3.4
Ahringer Feeding RNAi: rga-3 Source BioScience WB Clone: sjj_K09H11.3
Ahringer Feeding RNAi: cgef-1 Source BioScience WB Clone: sjj_C14A11.3
Feeding RNAi: mlc-4 (Redemann et al., 2010) N/A
Feeding RNAi: control(ctl) This paper N/A
PH-GBP gBlock (sequence on request) IDT DNA N/A
ctl (RNAi): gBlock (sequence on request) IDT DNA N/A

Software and Algorithms

Matlab Mathworks R2016a
Kilfoil Feature Tracking (feature2D.m) http://people.umass.edu/kilfoil/downloads.html N/A
Fiji (ImageJ) https://fiji.sc/# N/A
ActivityBase IDBS N/A

Please cite this article in press as: Rodriguez et al., aPKC Cycles between Functionally Distinct PAR Protein Assemblies to Drive Cell Polarity, Developmental Cell (2017), http://dx.doi.org/10.1016/j.devcel.2017.07.007
(pC1B-Ø) using SpeI and StuI to generate pC1B-pkc-3. Both plasmids were introduced by biolistic bombardment into HT1593 worms (Pratish et al., 2001), yielding NWG0012 and NWG0016.

The membrane-tethered GFP-binding protein (PH-GGBP) was generated by combining amino acids 1-175 corresponding to the PH domain of rat PH-PLCα1 (Audhya et al., 2005) and VHH4GBP (Caussinus et al., 2011) coupled by a SGQGSGGSGGS linker. The resulting sequence was codon-optimized (CAI = 0.49) and a single GFP intron inserted as described (Redemann et al., 2011). A synthetic gBlock (IDT DNA) encoding the PH-GGBP was PCR amplified and cloned in frame with a C-terminal codon-optimized mKate2 under the control of the m5x-5 promoter and nmy-2 3’ UTR in a MosSCI vector containing wild-type unc-119 obtained from the Hyman Lab. The resulting plasmid (pNG0018) was inserted at the tti5605 mos1 site locus of DP38 worms via CRISPR after mutating the sgRNA/PAM site following the method described (pNG0019) (Dickinson et al., 2013). Modified worms were crossed into DR466 to generate a stable male line expressing PH-GGBP (NWG0047). To rescue membrane localization of PAR-3 variants, we crossed NWG0047 with KK1216 (par-3::gfp) or KK973 (par-3::cr1::gfp) lines. We were unable to obtain a stable homozygous line for the endogenously tagged PAR-3::GFP, presumably due to the toxic effects of continuously targeting all PAR-3 to the membrane throughout embryogenesis. Thus, we used F1 progeny heterozygous for PAR-3::GFP for analysis. By contrast, for PAR-3::CR1::GFP, which is expressed ectopically from a multi-copy random insertion, we readily obtained animals homozygous for both PAR-3::CR1::GFP and PH-GGBP, which were used for subsequent analysis. However, no significant difference in the segregation of PAR-3::CR1::GFP was seen between heterozygous and homozygous animals.

For analysis of GFP::CDC-42, SA131 was used unless otherwise indicated.

For analysis of the effects of CDC-42(Q61L) on GFP::PKC-3 localization, zygotes were taken from F1 animals resulting from crossing KK1228 with either TH159 or WH423 due to difficulties obtaining stable animals homozygous for both markers.

**Cell Lines**

HEK-293 are female and were obtained from Cell Production, Cancer Research UK (CRUK) and cultivated in DMEM (Dulbecco’s modified Eagle’s medium), 10% FBS (fetal bovine serum) and penicillin–streptomycin (Invitrogen) (Kjær et al., 2013).

**Bacterial Strains**

OP50 bacteria and HT115(DE3) were obtained from CGC. DH5x was obtained from Colin Dolphin. Feeding by RNAi used HT115(DE3) bacteria strains containing a plasmid carrying the indicated RNAi feeding plasmid.

**METHOD DETAILS**

**C. elegans - RNAi Culture Conditions**

RNAi by feeding was performed similar to described methods (Kamath et al., 2003). Briefly, HT115(DE3) bacterial feeding clones were inoculated from LB agar plates to LB liquid cultures and grown overnight at 37°C in the presence of 10 μg/ml carbenicillin. Bacterial cultures were induced with 5 mM IPTG at 37°C for 4h with agitation before spotting 100 μl of induced bacteria onto 60 mm agar plates (10 μg/ml carbenicillin, 1 mM IPTG). L4 larva were added to RNAi feeding plates and incubated for 24-72 hr depending on gene and temperature. For temperature sensitive lines, feeding was performed at 15°C for 48-72 hr and shifted to 25°C for 2-5 hr before imaging. For double depletion experiments, L3/L4 larva carrying par-1(ze310) or par-2(iti5) temperature sensitive mutants were placed on RNAi plates at 15°C for 24 hr before a fraction of those were moved to fresh RNAi expressing plates for 18 to 22 hr at 25°C. Partial RNAi for mic-4 was performed for 14-24 hr at 20°C. For partial depletion of perm-1, rga-3/4 or pkc-3, bacteria expressing the desired clone were mixed at the indicated ratios with bacteria expressing control RNAi. par-3, par-6, cdc-42, pkc-3, perm-1, rga-3/4, cgef-1 and emb-27 clones are from the Ahringer library (Kamath et al., 2003), mic-4 is from Redemann et al., 2010. A control RNAI clone was generated by synthesizing a random 500bp sequence using the Matlab random number generator with no homology to the worm genome, cloned into Bgl-II / HindIII sites of L4440 (Addgene, plasmid#1654), and transformed into HT115 bacteria.

**C. elegans Embryos – Western Blots**

Embryos were obtained by a standard bleaching protocol and resuspended in NuPAGE LDS sample buffer (Invitrogen) prior to sonication using the Biorupture (Diagenode) for 5 - 30 s on, 30 s off cycles. Samples were heated at 70°C for 10 min before centrifugation at 13.000 rpm for 20 min to obtain cleared supernatant. Samples were run on a 12% NuPAGE gel using MOPS SDS running buffer (Invitrogen) and transferred onto PVDF membrane (Immobilon-P membrane 0.45 um, Millipore). PKC-3 and tubulin was detected using the primary (anti-PKC-3 1:10.000 and anti-tubulin 1:20.000) and secondary antibodies (as recommended by provider) indicated in the Key Resources Table and detected via chemiluminescence (ECL prime, GE Healthcare Life Sciences). PKC-3 band intensity was analyzed using the Fiji Gel analysis tool.

**C. elegans Zygotes – Drug Treatment**

All drug treatment experiments were performed in 10 to 50% perm-1(RNAi) (Carvalho et al., 2011). Drugs were dissolved in DMSO and used at the following concentrations: phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, P1585-1MG), 100 μM; CRT90 (CRT0103390, Cancer Research Technology LTD), 10 μM. When drug treatment alone was required, we obtained zygotes with permeable eggshells by placing L4 animals on a 1:1 mix of bacteria expressing perm-1(RNAi) and ctl (RNAi) for 16 to 20 hr at
20°C. When drug treatment was combined with additional RNAi treatment, L4 animals were placed on bacteria expressing \textit{perm-1(RNAi)} mixed at a 1:9 ratio with bacteria expressing the desired RNAi (par-3, par-6, cdc-42, control) and incubated for 40-48 hr at 20°C.

**C. elegans Zygotes - Immunofluorescence**

Immunofluorescence was performed as previously described (Andrews and Ahringer, 2007). Briefly, gravid hermaphrodite worms were washed and then transferred to a 7 μl drop of M9 on a 0.1% poly-lysine coated well. Embryos were released using a needle and then covered with a coverslip to compress the embryos. Slides were snap-frozen on dry ice for 30 min after which the coverslip was quickly removed and the slide fixed in methanol at room temperature for 20 min. Samples were washed and re-hydrated with PBS followed by 10 min washes in PBS+0.2% Tween-20 before proceeding with antibody incubations, DAPI staining and mounting in Mowiol (Sigma-Aldrich). All antibodies used in this study are listed in **Key Resources Table**. Primary antibody dilutions used: anti-PAR-2 1:500, anti-PAR-6 1:10, anti-PKC-3 1:500 and anti-PAR-3 1:50. Secondary antibodies were used as recommended by provider. Confocal images were acquired using Carl Zeiss Axioplan 2, LSM510 Meta confocal equipped with LSM image software and Nikon A1R equipped with Nikon elements software and a 63x objective. Cortical super-resolution images were acquired using the DeltaVision OMX system equipped with SoftWoRx and OMX acquisition software. Secondary processing of images was performed using Photoshop CS5 and Illustrator CS5 (Adobe).

**C. elegans zygotes - Live Imaging**

Embryos were dissected in 2-4 μl of M9 buffer (22 mM KH₂PO₄, 42 mM NaHPO₄, 86 mM NaCl and 1 mM MgSO₄) on a coverslip and mounted under 2% agarose pads (Zipperlen et al., 2001) or dissected in Shelton’s Growth Medium (Edgar and Goldstein, 2012) and mounted with 16-21 μm polystyrene beads between a slide and coverslip and sealed with VALAP (Goehring et al., 2011a). 16-18 μm beads were used for cortex imaging to maximize imaging surface. In all other cases, 21 μm beads were used to minimize compression effects on development. For CRT90 experiments, embryos were dissected in the presence of 10 μM CRT90. For C1B targeting experiments, two sides of the coverslip were left unsealed to create a flow chamber (Goehring et al., 2011a) and PMA washed in at the indicated times.

To maximize viability, embryos were typically imaged at 20-22°C, except for temperature sensitive alleles, which were imaged at the indicated temperatures using an objective temperature control collar (Biotools / Linkam, PE94). For consistency, establishment phase embryos were taken at pronuclear meeting, and maintenance phase was defined as the interval from nuclear envelope break-down to metaphase.

Cortex images were captured with a 100x 1.49 NA TIRF objective on a Nikon TIE (Nikon) equipped with an iLas2 TIRF unit (Roper), 488 or 561 fiber coupled diode lasers (Obis), and an Evolve Delta camera (Photometrics). Midplane imaging was performed on Carl Zeiss Axioplan 2, LSM510 Meta confocal or a Nikon TIE with 63x or 100x objectives, further equipped with either a Spectra-X LED light source (wide-field) or a custom X-Light V1 spinning disk system (CrestOptics, S.p.A.) with 50 μm slits, 488, 561 fiber-coupled diode lasers (Obis) and either a CoolSnap HQ or Evolve Delta (Photometrics). Imaging systems were run using Metamorph (Molecular Devices) and configured by Cairn Research (Kent, UK).

**In vitro PKC Enzyme Assays**

The ability of compounds to inhibit the kinase activity of recombinant human baculovirus-expressed full-length PKCᵢ was measured using the IMAP fluorescence polarization (FP) progressive binding system (Molecular Devices #R8127, Sunnyvale, CA) in 384-well black, non-binding, flat-bottom assay plates (Corning #3575, Corning, NY). The assay mixture (final volume = 10 μL) contained 20 mM Tris-HCl (pH 7.5), 150 μM ATP, 10 mM MgCl₂, 0.01% Triton X-100, 250 μM EGTA, 1 mM DTT, 15 pm M PKCᵢ (EMD Millipore #14-505, Billerica, MA), 100 nM FAM-PKCᵢ-pseudosubstrate (Molecular Devices #RP7548), 0.1% DMSO and various concentrations of test compound. Compounds were diluted in 100% DMSO and added to the assay plate at 100 nL using the BioMek NX pin tool (Beckman Coulter, Indianapolis, IN). Enzyme reactions were initiated by the addition of ATP (Sigma-Aldrich #A7699, St. Louis, MO), followed by incubation of the plates for 1 hour in a 25°C incubator. A 20 μL aliquot of IMAP detection reagent (1:400 in 85% 1X Binding Buffer A and 15% 1X Binding Buffer B) was added to each well followed by a 2-hour incubation at 25°C. Fluorescence polarization was then measured using the PerkinElmer Envision 2102 multi-label plate reader (PerkinElmer, Waltham, MA) using the FP dual mirror, FP480 excitation filter and P-pol 535 and S-pol 535 emission filters. Data analysis was performed using ActivityBase (IDBS, Guilford, UK). I₅₀ values were calculated by plotting percent inhibition versus log10 of the concentration of compound and fitting to the 4-parameter logistic model (top and bottom constrained to 100 and 0, respectively) in XLFit 4 (IDBS).

The PKCᵢᵡ kinase assay was performed using the IMAP FP progressive binding system as described above for PKCᵢ but with some modifications. The concentration of PKCᵢᵡ (recombinant active protein, His tagged, expressed in Sf21 cells, Millipore, 14-525) was 10pM, while the substrate concentrations were 100 nM and 40 μM for the FAM-PKCᵢᵡ-pseudosubstrate (Molecular Devices #RP7548) and ATP, respectively.

**Cellular Biochemical Assay**

HEK-293 cells were transfected in a 10 cm dish as per the manufacturer’s instructions (Corning). After 16 hr, the cells were trypsinized and seeded into a 96-well plate at 1.5 × 10⁴ cells/well and medium was replenished. After a further 24 hr, the medium was replaced by new medium and a range of CRT0103390 inhibitor concentrations. After 1 hr of inhibitor treatment, lysates were prepared using
ice-cold Tris lysis buffer [150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA and 1% Triton X-100]. Lysates were transferred on to an anti-FLAG-coated ELISA plate (Sigma) and incubated for 2 hr with gentle shaking, followed by an automated wash step (Tecan plate washer) with wash buffer [50 mM Tris (pH 7.5), 0.15 M NaCl and 0.02% Tween 20]. The immunocomplexed protein was incubated with anti-pLLGL1/2 (S650/S654) primary antibody overnight at 4°C, followed by an automated wash and then addition of HRP-conjugated secondary antibody. After a further wash, 3,3',5,5'-tetramethylbenzidine (Sigma) was added according to the manufacturer’s instructions and attenuation was read at 450 nm using an Ascent plate reader (Thermo Labsystems).

**Kinase Selectivity**
CRT0103390 was profiled using the KINOMEScan in vitro competition binding screening platform at DiscoveRx against a panel of 442 mutant and non-mutant kinases at a test concentration of 1 μM. Selectivity scores were calculated as the number of non-mutant kinases with % activity relative to control < 20/number of non-mutant kinases tested. CRT0103390 demonstrated a high degree of selectivity in this panel, with an S(80) of 0.09.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Image Analysis - General**
All image analysis was performed in Fiji (ImageJ) and Matlab (Mathworks).

**Image Analysis - Asymmetry Index (ASI)**
The asymmetry index (ASI) of a feature is defined by:

$$\frac{A - P}{2(A + P)}$$

where A and P define the anterior and posterior signal, respectively. Raw ASI values are normalized to the mean ASI observed in respective controls, such that a value of 1 indicates wild-type asymmetry and zero indicates complete loss of asymmetry. Anterior and posterior signals are defined depending on the condition examined and include cross-sectional area (AB vs P1 asymmetry), fluorescence intensity on the two cell halves for the membrane (midsection PAR analysis) or cytoplasm (MEX-5, PIE-1), or cluster number (CHIN-1).

**Image Analysis – Cluster Index**
The Cluster Index is defined as the variance in cortical intensity within the anterior domain. It was calculated in Matlab across user specified ROIs that were subject to background subtraction and normalization to mean intensity before analysis.

**Image Analysis – CHIN-1 Foci**
CHIN-1 foci were identified using the feature2D.m script, part of the feature detection and particle tracking package from the Kilfoil Lab (Pelletier et al., 2009). Embryos were automatically detected and partitioned into 3 domains (Anterior, Middle, Posterior) and normalized anterior vs posterior particle densities used for ASI calculation.

**Image Analysis – MEX-5 Mobility**
For MEX-5 mobility, five pre-bleach frames were captured by spinning disk confocal microscopy. A central 20-pixel wide stripe was then bleached along the AP axis using a 473 diode laser (Obis) and recovery was monitored every 2 s. Because MEX-5 is uniform in the quantified conditions, fluorescence was monitored within a central 20 x 100-pixel box.

**Image Analysis – Colocalization**
Colocalization analysis of PAR-6 and PAR-3 was performed in ROIs at the anterior cortex of establishment phase zygotes (wild-type n=8 and pkc-3(ts) n=9). Costes’ Mask and intensity correlation quotient (Li et al., 2004) were obtained using JaCOP plug-in in Fiji.

**Image Analysis – Flow Speeds**
Anterior-directed cortical flow speed during establishment phase was measured using midplane, brightfield images acquired every second until late establishment phase at which point we switched to fluorescent imaging to obtain suitable images for measuring PAR asymmetry. Using the Kymograph Plugin in Fiji (Seitz and Surrey, 2006), we generated kymographs for individual embryos by tracing a segmented line along the cortex starting at the origin of flow. A minimum of 10 yolk granule trajectories, each spanning approximately 200s were selected for a minimum of 5 embryos per condition. The cortical flow velocity was defined as the total distance over time calculated from a line connecting the start and end positions of the granule on the kymograph. Measurement of cortical flow in wild-type embryos expressing GFP fusions to PAR-3, PKC-3 or CDC-42 yielded a velocity of 7.1+/−1.4 μm/min (n=22), consistent with previously published values (7.66+/−1.0 μm/min, n=6)(Munro et al., 2004).
Image Analysis – Anterior Cortical Intensity (Immunofluorescence)
Anterior cortical intensity of each PAR protein is the mean greyscale value of a line 2 pixels wide (in each corresponding fluorescent channel) covering the PAR-3 cortical domain of the zygote. The cortical intensity value is then normalized by dividing it by the mean greyscale value of a nearby cytoplasmic region to correct for embryo IF staining variability. In par-3(RNAi) zygotes, we cannot distinguish anterior from posterior cortex and the entire cortex of zygotes is analyzed. Each experimental condition was analyzed in three independent experiments.

Image Analysis – Anterior PAR Retraction (Immunofluorescence)
The posterior boundary of anterior PARs in fixed, midsection fluorescent images is defined at the intersection between the equatorial zygote line (longest line linking the zygotes’ poles) and the line that links the cortical ends (top and bottom) of the PAR protein analyzed. Retraction is the distance between this posterior boundary and the posterior pole of the zygote. Retraction difference is defined as the difference in retraction distance between PAR-3 and PKC-3. Data were collected from three independent experiments.

Image Analysis – Intensity Profile Extraction
In general, to assess PAR signal from midsection images, a 60-pixel wide stripe encompassing the cell membrane was extracted and straightened to generate a profile for each embryo for further analysis.

For spatial analysis (ASI, profile plots, domain size, segregation efficiency), the top 4 central pixels corresponding to the membrane were taken at each x-position and averaged to the given local membrane signal. Background and cytoplasmic signal were calculated locally from inner and outer edges of this stripe, allowing normalization for variation in signal between conditions. Briefly, background was subtracted and then membrane divided by cytoplasmic intensity.

Image Analysis – PAR-3 versus PKC-3 Cortical Profile Comparison (Immunofluorescence)
To quantitatively compare PAR cortical profiles in multi-labelled fixed embryos, two identical profiles along the cortex were extracted in each channel as above. Each set of profiles was split in half to generate two boundary regions. After normalization to maximum and minimum values, profiles were registered using the inflection point, c, based on fitting each PAR-3 profile using the following function:

\[ I(x) = a + \frac{b}{2} \left( \text{erf} \left( \frac{mx - c}{c'} \right) \right) \]

where erf is the error function, c is the domain boundary position, m the boundary slope and a and b allow for scaling and displacement on the y-axis.

Image Analysis – Domain Size/Segregation Efficiency
To extract domain size data (PAR-2 domain size change, segregation efficiency) from single channel images, cortical fluorescence profiles were normalized to total embryo perimeter length and aligned to the center of posterior PAR domain determined by fitting the profile to the following function:

\[ I(x) = a \pm \frac{b}{2} \left( \text{erf} \left( \frac{mx - c_1}{c_2} \right) - \text{erf} \left( \frac{mx - c_2}{c_1} \right) \right) \]

with the center of the posterior domain specified by:

\[ \frac{c_2 - c_1}{2} \]

posterior domain size given by:

\[ \frac{c_2 - c_1}{L} \]

and anterior domain size given by:

\[ 1 - \frac{c_2 - c_1}{L} \]

where L is the length of the profile.

PAR-2 domain size change for each embryo was calculated as the ratio of domain size taken from images before and 5 min after PMA addition. Segregation efficiency into the anterior was scored by relative anterior domain size, with smaller anterior domains defined as more efficient segregation.

Image Analysis – Total Membrane Signal Change
To estimate total membrane signal in PAR-6 and PKC-3 rescue experiments straightened profiles were projected in x to give a cross-section profile spanning background, crossing the membrane and into the cytoplasm for the full circumference. Cross-section profiles were then normalized with background = 0 and cytoplasm = 1. To get the most accurate estimate of isolated membrane signal,
we generated a mean cross-section for embryos with no detectable membrane signal from par-3 and par-6 RNAi embryos to define the shape of the outside to inside fluorescence step. The shape of this curve was extremely consistent allowing a mean profile to be generated, which could then be subtracted from individual embryo cross-section profiles with the sum of the difference taken as membrane signal.

For PAR-2 retention in C1B-induced PKC-3 membrane-targeting experiments, a cytoplasm-normalized 4-pixel wide stripe encompassing the cell membrane was taken from images before and 5 min after PMA addition to generate profiles. PAR-2 retention was defined as the ratio of total membrane signal before and after PMA addition.

Statistics
For all assays, significance was assessed using an unpaired, two-tail Student’s T test unless otherwise noted with the following criteria: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean values plus all data points or mean ± 95% confidence interval (CI), in which case (N) is indicated.

Data and Software Availability
Sequence data for pkc-3(ne4246) has been submitted to Wormbase (WB Gene: pkc-3).