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Article

aPKC Phosphorylates Miranda to Polarize Fate Determinants during Neuroblast Asymmetric Cell Division

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Summary

Background: Asymmetric cell divisions generate daughter cells with distinct fates by polarizing fate determinants into separate cortical domains. Atypical protein kinase C (aPKC) is an evolutionarily conserved regulator of cell polarity. In *Drosophila* neuroblasts, apically restricted aPKC is required for segregation of neuronal differentiation factors such as Numb and Miranda to the basal cortical domain. Whereas Numb is polarized by direct aPKC phosphorylation, Miranda asymmetry is thought to occur via a complicated cascade of repressive interactions (aPKC –| Lgl –| myosin II –| Miranda).
Results: Here we provide biochemical, cellular, and genetic data showing that aPKC directly phosphorylates Miranda to exclude it from the cortex and that Lgl antagonizes this activity. Miranda is phosphorylated by aPKC at several sites in its cortical localization domain and phosphorylation is necessary and sufficient for cortical displacement, suggesting that the repressive-cascade model is incorrect. In investigating key results that led to this model, we found that Y-27632, a Rho kinase inhibitor used to implicate myosin II, efficiently inhibits aPKC. Lgl3A, a nonphosphorylatable Lgl variant used to implicate Lgl in this process, inhibits the formation of apical aPKC crescents in neuroblasts. Furthermore, Lgl directly inhibits aPKC kinase activity.
Conclusions: Miranda polarization during neuroblast asymmetric cell division occurs by displacement from the apical cortex by direct aPKC phosphorylation. Rather than mediating Miranda cortical displacement, Lgl instead promotes aPKC asymmetry by regulating its activity. The role of myosin II in neuroblast polarization, if any, is unknown.

Introduction

The presence of molecularly distinct cortical domains is a hallmark of many cell types [1, 2]. In epithelial cells, apical and basolateral domains mediate junction formation and transport [2]. *Drosophila* neuroblasts use polarized cortical domains during mitosis to generate progeny that assume different developmental fates [3, 4]. Early in mitosis, fate determinants that specify neuroblast fate form an apical cortical domain whereas those that specify differentiation form a basal domain, and these domains are ultimately segregated into separate daughter cells [5]. In these cases and many others, a polarized cell cortex is essential for proper function. Although polarity is a fundamental characteristic of diverse cell types, many questions concerning its molecular origins remain.

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The atypical protein kinase C (aPKC) is a key regulator of cell polarity [6]. In embryonic neuroblasts, aPKC localizes to the apical cortex, where it restricts factors that specify basal cell fate, such as Miranda and Numb, to the basal cortex [5, 7, 8]. Recruitment of aPKC to the apical neuroblast cortex occurs through the combined action of Bazooka (Baz, also known as Par-3), Par-6, and Cdc42 [7–9]. Apically localized Baz recruits GTP-bound Cdc42, which in turn binds the semi-CRIB domain of Par-6 to recruit aPKC [6].

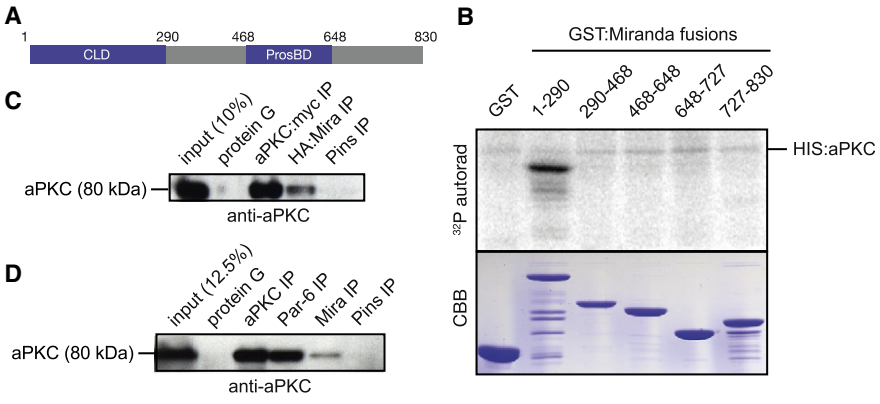
Although aPKC apical recruitment is fairly well understood, the mechanisms by which aPKC activity is translated into the polarization of downstream components are poorly defined. Although Numb has recently been identified as an aPKC substrate [10] and phosphorylation leads to its cortical displacement, the mechanism of Miranda polarization is thought to be much more complex. Apical aPKC phosphorylates the tumor suppressor Lgl [11, 12], a candidate myosin II inhibitor [13]. Myosin II is thought to physically displace Miranda from the cortex, "pushing" it from the apical to basal cortex [14]. This leads to a complex "repressive cascade" pathway in which aPKC phosphorylates Lgl, preventing its inhibition of myosin II, ultimately removing Miranda from the cortex at sites of aPKC activity. However, the mechanisms by which Lgl might inhibit myosin II and how myosin II displaces Miranda from the cortex are unknown. Furthermore, several key observations are inconsistent with this model, including the cortical association of Miranda in *lgl aPKC* double mutants [5].

Here we examined the mechanism by which aPKC polarizes Miranda and found that Miranda is an aPKC substrate. Via a Miranda cortical displacement assay in cultured S2 cells, we identified several Miranda phosphorylation sites that are responsible for its removal from the cortex. These results, along with expression of nonphosphorylatable Miranda mutants in neuroblasts, lead us to conclude that phosphorylation is necessary and sufficient for cortical displacement, inconsistent with the repressive-cascade model. By examining key pieces of evidence that support this model, we found that a Rho kinase inhibitor used to implicate myosin II [14, 15] is nonspecific and efficiently inhibits aPKC at concentrations well below those used in previous studies. A nonphosphorylatable Lgl variant used to place Lgl between aPKC and Miranda [12] inhibits aPKC apical crescent formation. Furthermore, Lgl directly inhibits aPKC kinase activity. Based on these observations, we propose that aPKC directly phosphorylates Miranda to yield mutually exclusive cortical domains and that Lgl's role in polarity is to restrict aPKC activity to the apical cortex.

Results

Miranda Is an aPKC Substrate

Recently, aPKC was shown to phosphorylate the cell fate determinant Numb [10]. To determine whether Miranda might also be an aPKC substrate, we expressed and purified several Miranda truncations and incubated them with recombinantly purified aPKC. Miranda contains an NH_2-terminal cortical localization domain (residues 1–290) [16–18] that is responsible for cortical targeting in neuroblasts, and we observed



E

MSFSKAKLKR FNDVDVAICG SPAASNSSAG SAGSATPTAS SAAAAPPTVQ
PERKEQIEKF FKDAVRFASS SKEAKEFAIP KEDKKSKGLR LFRTPSLPQR
LRFRPTPSHT DTATGSGSGA STAASTPLHS AATTPVKEAK SASRLKGKEA
LQYEIRHKNE LIESQLSQLD VLRRHVDQLK EAEAKLREEH ELATSKTDRL
IEALTSENLS HKALNEQMGQ EHADLLERLA AMEQQLQQQH DEHERQVEAL
VAESEALRLA NELLQTANED RQKVEEQLQA QLSALQADVA

Figure 1. aPKC Binds and Phosphorylates Miranda

(A) Miranda domain architecture.

(B) The Miranda cortical localization domain is specifically phosphorylated by aPKC. Phosphorimaged and Coomassie-stained SDS gels of GST and GST:Miranda fusion proteins in the presence of recombinant HIS:aPKC and ^{32}P are shown. GST:Miranda 1–290 is phosphorylated, whereas all other constructs are not. A Coomassie-stained gel is shown as a loading control.

(C) Miranda interacts with aPKC in transfected S2 cells. Immunoblot of S2 cell lysates from cells transfected with aPKC:myc and HA:Miranda immunoprecipitated with anti-aPKC, anti-HA, or anti-Pins antibodies is shown. Protein G and anti-Pins antibody were used as controls.

(D) Miranda interacts with aPKC in *Drosophila* embryonic extracts. Immunoblot of embryonic lysates immunoprecipitated with anti-aPKC, anti-Par-6, anti-Miranda, or anti-Pins antibodies is shown. Protein G and anti-Pins antibody were used as controls.

(E) Identification of residues within Miranda 1–290 phosphorylated by aPKC in vitro. Lavender-highlighted residues indicate coverage by liquid chromatography-tandem mass spectrometry, red lettering indicates residues identified as phosphorylated, and asterisks indicate residues tested by point mutation as in Figure 2. Phosphorylation prediction programs predicted residues 141 and 143 to be highly likely for phosphorylation by aPKC.

phosphorylation specific to this region (Figures 1A and 1B). Consistent with Miranda being an aPKC substrate, Miranda coimmunoprecipitates with aPKC from transfected S2 cells (Figure 1C) and *Drosophila* embryonic extracts (Figure 1D), indicating that the two proteins interact with one another in vivo.

We identified the sites within Miranda that are phosphorylated by aPKC via mass spectrometry. Tandem mass spectrometry of recombinantly purified Miranda 1–290 incubated with recombinant aPKC followed by trypsin digestion yielded nearly complete coverage of the cortical localization domain (Figure 1E). Analysis of these tryptic fragments yielded several groups of phosphorylated residues within the Miranda cortical localization domain (Figure 1E).

Miranda Phosphorylation by aPKC Is Necessary and Sufficient for Cortical Displacement

To rapidly determine which phosphorylation sites within Miranda may be responsible for cortical displacement, we developed a cortical displacement assay in cultured *Drosophila* S2 cells. We quantitatively assayed cortical-to-cytoplasmic signal ratios of transiently transfected Miranda, which is heavily enriched at the cortex of S2 cells (Figures 2A and 2S; see also Figure S1 available online), similar to the basal enrichment of Miranda at the cortex of metaphase neuroblasts. The Miranda neuroblast cortical localization domain is sufficient for cortical localization in S2 cells (Figures 2B and 2S), suggesting that the association mechanism in neuroblasts is also utilized in S2 cells. Expression of aPKC with Miranda leads to loss of cortical staining and an increase in cytoplasmic signal (Figures 2C and 2S). Kinase activity is required for this effect, because a kinase-dead aPKC variant does not displace Miranda (Figures 2D and 2S). We conclude that aPKC removes Miranda from the S2 cell cortex in a manner similar to neuroblasts, and that this system can be used as a model for investigating aPKC-dependent Miranda cortical displacement.

To identify phosphorylation sites that might be coupled to Miranda cortical association, we examined the localization behavior of Miranda variants in which the in vitro phosphorylation sites had been changed to alanine and tested whether these proteins could be displaced into the cytoplasm by aPKC. All Miranda variants localized to the cortex in the absence of aPKC (Figures 2E–2J). Mutation of residues 69, 70, and 71 (Mira69AA) or residues 141 and 143 (Mira141AA) did not have any appreciable effect on cortical exclusion by aPKC (Figures 2K, 2L, and 2T). However, mutation of residue 96 (Mira96A), residues 194 and 195 (Mira194AA), or residues 205 and 206 (Mira205AA) strongly prevented Miranda displacement by aPKC (Figures 2M–2O and 2T). We combined the mutated residues that reduced aPKC's ability to displace Miranda from the cortex into one protein (Mira5A) and assayed its localization in the presence of aPKC. Mira5A remains cortical in 90% of cells when expressed with aPKC, indicating that these sites regulate cortical exclusion of Miranda (Figures 2P and 2T). We conclude that aPKC phosphorylation of Miranda is necessary for excluding Miranda from the cell cortex of S2 cells.

We next tested whether phosphorylation by itself can lead to Miranda displacement from the S2 cell cortex. We generated aspartic acid mutations at sites of aPKC phosphorylation and examined the localization of the phosphomimetic protein in the absence of aPKC. Mutation of residue 96 (Mira96D) or residues 205 and 206 (Mira205DD) caused Miranda to become cytoplasmic even in the absence of aPKC, indicating that phosphorylation is sufficient for cortical exclusion (Figures 2Q, 2R, and 2T). Thus, based on the results of both alanine and aspartic acid mutations, we conclude that phosphorylation is necessary and sufficient for excluding Miranda from the cortex of S2 cells.

We verified that Miranda phosphorylation leads to cortical displacement in neuroblasts by using transgenic flies expressing Mira5A. We expressed wild-type Miranda and Mira5A in larval neuroblasts via the *prospero-GAL4* driver and assayed for Mira5A localization and neuroblast polarity. Mira5A was unpolarized in mitotic larval neuroblasts, in stark contrast to the basal enrichment observed in wild-type neuroblasts (Figures 3A–3C and 3G). To determine whether nonphosphorylatable Miranda is cortically enriched throughout development, we

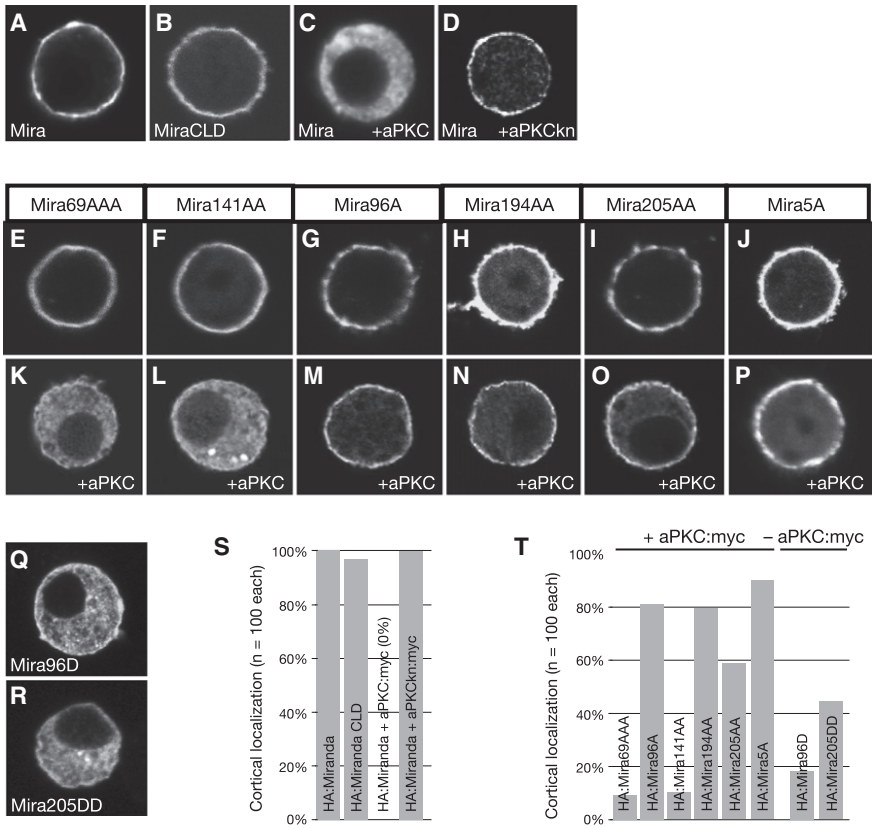


Figure 2. aPKC Is Necessary and Sufficient to Displace Miranda from the S2 Cell Cortex

Expression of Miranda and aPKC in fixed S2 cells stained the by indicated markers. n = 100 cells for all quantifications.

(A) Miranda localizes to the S2 cell cortex (100%, scored as in Figure S1).

(B) The Miranda neuroblast cortical localization domain is also sufficient for S2 cortical localization (96%).

(C and D) Miranda is displaced into the cytoplasm when expressed with aPKC (0% cortical) (C), but not when expressed with aPKCkn, a variant that lacks kinase activity (100% cortical) (D).

(E–J) All Miranda variants with alanine mutations localized to the cortex in the absence of aPKC.

(K and L) Miranda with alanine mutations in residues 69, 70, and 71 (Mira69AAA) localizes predominantly to the cytoplasm in the presence of aPKC:myc (92%) (K), as does a construct with alanine mutations in residues 141 and 143 (Mira141AA) (90%) (L).

(M–O) Miranda with alanine mutations in residue 96 (Mira96A) is predominantly cortical in the presence of aPKC:myc (82%) (M), as are constructs with alanine mutations in residues 194 and 195 (Mira194AA) (79%) (N) or in residues 205 and 206 (Mira205AA) (58%) (O).

(P) Miranda with alanine mutations in all five residues showing substantial effects (Mira5A; residues 96, 194, 195, 205, and 206) localizes predominantly to the cortex in the presence of aPKC:myc (90%).

(Q and R) Miranda with aspartic acid mutations at residues 96 (Mira96D) (82%) (Q) or at residues 205 and 206 (Mira205DD) (55%) (R) is predominantly cytoplasmic in the absence of aPKC:myc.

(S and T) Quantification of Miranda constructs and point mutants in the presence or absence of aPKC:myc.

expressed Mira5A in embryonic neuroblasts via the *worniu-GAL4* driver and found similar uniform cortical localization (Figures 3D and 3G). In fact, Mira5A localized uniformly cortical throughout mitosis (Figures 3E and 3F). The presence of Mira5A had no effect on apical complex formation, because Par-6 and aPKC correctly localized to the apical cortex (Figures 3B–3D and 3G). Mira5A is functional, retaining the ability to bind the cell fate determinant Prospero, as indicated by the uniformly cortical localization of Prospero in neuroblasts expressing Mira5A (Figure 3B″). In addition, Mira5A expression was not able to rescue embryonic lethality of *miranda*^{ZZ176} animals [16], although a wild-type form is able to do so [19], indicating that phosphorylation and proper polarization of Miranda by aPKC are necessary for normal development (data not shown). We conclude that Miranda phosphorylation by aPKC is necessary and sufficient for Miranda cortical displacement.

The Rho Kinase Inhibitor Y-27632 Inhibits aPKC

The observation that phosphorylation of Miranda by aPKC is necessary and sufficient for cortical displacement leads to a dramatic simplification of the repressive-cascade model: Miranda polarity occurs by a mechanism in which cortical regions containing aPKC activity displace Miranda by direct phosphorylation. Is it possible to explain the previous observations that gave rise to the repressive-cascade model in terms of this simpler mechanism?

Myosin II was thought to "push" Miranda from the apical cortex of neuroblasts, leading to basal enrichment [14]. Much of the evidence for myosin II involvement in aPKC-mediated cortical displacement of Miranda in the repressive-cascade model resides on the observation that Miranda polarity is lost in neuroblasts from embryos injected with the Rho kinase inhibitor Y-27632 [14, 15]. Rho kinase phosphorylates the myosin II regulatory light chain (RLC), which induces filament assembly and motor activity [20]. Because the uniform cortical Miranda localization in Y-27632-treated neuroblasts is identical to *apkc* neuroblasts and kinase inhibitors are often not entirely specific, we hypothesized that the inhibitor phenotype results from inhibition of aPKC. (In fact, inhibition of canonical PKC was noted in the original description of Y-27632 [21].) By using an in vitro kinase assay with purified, recombinant aPKC, we found that Y-27632 efficiently inhibits aPKC with an IC_{50} < 10 μM (Figure 4A). The concentration of this compound used to examine its effect on embryonic neuroblast polarity (~50 mM Y-27632 [14, 15]) vastly exceeds the IC_{50} of aPKC and Rho kinase (K_i ~ 0.1 μM [21]), indicating that aPKC was likely to have been inhibited in these experiments. Given the cross-reactivity of Y-27632, the loss of Miranda polarity can be explained by direct inhibition of aPKC.

As the S2 cell system accurately recapitulates aPKC-mediated cortical displacement of Miranda, we also examined the effect of myosin II on Miranda localization in S2 cells. Although we were unable to reduce myosin II levels sufficiently via RNAi, we tested whether myosin II behaves as predicted by the repressive-cascade model. This model predicts that cells expressing sufficient aPKC to displace Miranda should alter

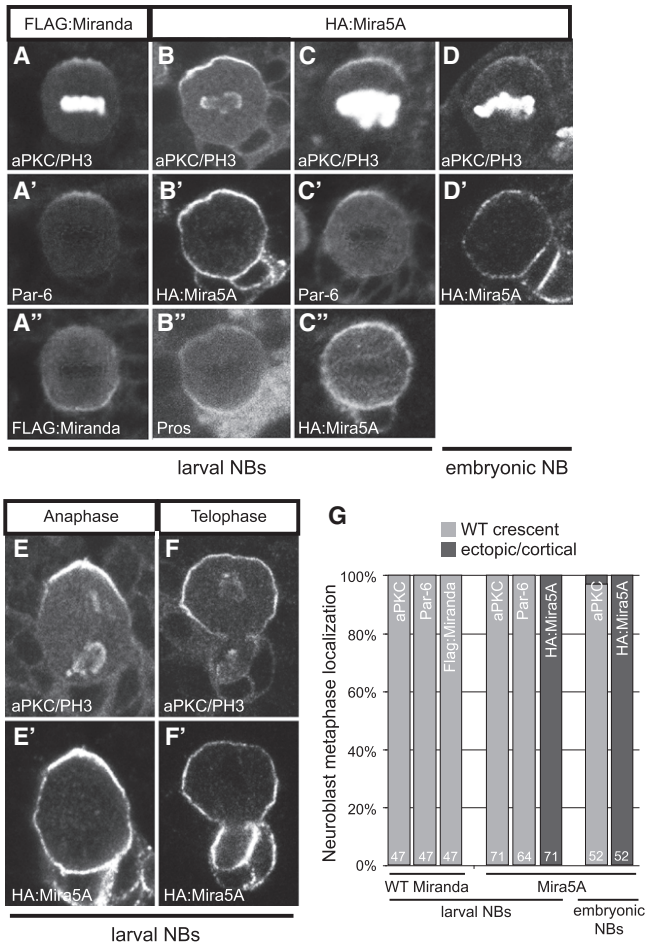


Figure 3. Phosphorylation of Miranda Is Necessary for Exclusion from the Neuroblast Apical Cortex at Metaphase

(A–F) An unphosphorylatable Miranda mutant is depolarized in embryonic and larval neuroblasts. Expression of *UAS* transgenes by *prospero-GAL4* in brains at 96 hr after larval hatching (ALH) or by *worniu-GAL4* in embryos at stages 11–13 stained by the indicated markers is shown.

(A) Wild-type FLAG:Miranda, used as a control, localized to the basal cortex (100%; n = 47), whereas aPKC and Par-6 localized to the apical cortex (100%; n = 47).

(B–F) HA:Mira5A was not restricted to the basal cortex and localized uniformly cortical in larval neuroblasts (100%; n = 71) and embryonic neuroblasts (100%, n = 52) during metaphase (B–D) and throughout mitosis (E and F).

(B–D) aPKC (larval: 100%, n = 71; embryonic: 96%, n = 52) and Par-6 (larval: 100%, n = 64) remained wild-type. As seen in (B″), Prospero mislocalized uniformly cortical in Mira5A-expressing neuroblasts.

(G) Quantification of neuroblast metaphase localization.

myosin II levels at the cortex [14]. Endogenous myosin II is predominantly cytoplasmic in S2 cells and enriches at the cleavage furrow during mitosis (Figures 4B, 4C, and 4H). However, we observed no appreciable change in cortical myosin II levels in cells expressing aPKC (Figures 4D and 4H). The model also predicts that myosin II physically displaces Miranda at sites along the cortex, suggesting competition for the same cortical binding sites. We attempted to directly displace Miranda via myosin II overexpression but were unable to do so with full-length heavy chain (Figures 4E and 4H; Zipper in *Drosophila*) or a heavy-chain fragment that localizes predominantly to the cortex (Figures 4F and 4H) [22], indicating that the presence of myosin II at the cortex is not sufficient to displace Miranda. Because active myosin II may be necessary to displace Miranda from the cortex, we expressed a phosphomimetic form of the myosin II RLC subunit *spaghetti squash* (*sqhEE*) with Miranda in S2 cells, but we again observed no change in cortical Miranda (Figures 4G and 4H). This protein also fails to have an effect in neuroblasts [14].

Lgl Directly Inhibits aPKC to Depolarize Miranda

In the repressive-cascade model, aPKC phosphorylation of Lgl is required for Miranda cortical displacement. This aspect of the model arose from the observation that neuroblasts expressing Lgl3A, a nonphosphorylatable variant, exhibit Miranda polarization defects [12, 14], which was interpreted to result from a requirement for Lgl phosphorylation in Miranda cortical release. To explore the nature of the Lgl3A phenotype more closely, we examined neuroblasts expressing Lgl3A driven by *worniu-GAL4.* Consistent with previous observations, the majority of neuroblasts exhibited ectopic cortical Miranda compared to wild-type (Figures 5A–5C). However, we also found that apical aPKC and Par-6 crescents are severely reduced compared to wild-type in neuroblasts with ectopic Miranda cortical localization (Figures 5B and 5C). We also examined the effect of Lgl3A on Miranda cortical displacement in S2 cells. Miranda and Lgl3A colocalize at the S2 cell cortex as expected (Figures 5D and 5F). However, when aPKC is also expressed, Lgl3A remains at the cortex but Miranda is displaced into the cytoplasm (Figures 5E and 5F), inconsistent with Lgl phosphorylation being a prerequisite for Miranda displacement. Thus, an alternative explanation of Lgl3A-induced Miranda depolarization is that it results from compromised aPKC activity.

We sought to determine whether the Lgl3A-mediated Miranda phenotype results from excessive inhibition of aPKC by Lgl3A or a requirement for Lgl phosphorylation in Miranda displacement. We examined Miranda localization in neuroblasts expressing Lgl3A along with an active, predominantly cytoplasmic aPKC (aPKCΔN) that no longer binds Lgl through Par-6 (the N-terminal portion of aPKC that binds Par-6 is missing [12]). If the Lgl3A phenotype results from inhibition of aPKC activity, aPKCΔN should overcome this inhibition and displace Miranda from the cortex, whereas if phosphorylation of Lgl is required for Miranda displacement, Miranda should remain cortical. We found that Miranda is efficiently driven into the cytoplasm of neuroblasts expressing Lgl3A and aPKCΔN (Figure 5G), further suggesting that Lgl phosphorylation is not required for aPKC-mediated Miranda cortical displacement. This observation is consistent with the cytoplasmic Miranda observed in neuroblasts expressing aPKC-CAAX (containing a lipid modification tag) and Lgl3A [5].

Although Lgl has been shown to inhibit aPKC [23], the mechanism of inhibition has been unclear. To test whether Lgl is capable of directly inhibiting aPKC in vitro, we incubated recombinantly purified aPKC with the cortical localization domain of Miranda and increasing concentrations of purified Lgl. Miranda was efficiently phosphorylated by aPKC in the absence of Lgl (Figure 5J). However, upon titration of Lgl, phosphorylation of Miranda was drastically reduced, indicating that Lgl can directly inhibit aPKC activity. Because Lgl is an aPKC substrate, it is possible that it competitively inhibits aPKC activity, although Baz is also an aPKC substrate and does not inhibit aPKC [23]. Thus, we conclude that Lgl directly regulates aPKC activity, resulting in a simplified model for cell fate determinant segregation wherein aPKC directly phosphorylates and displaces Miranda from the neuroblast cortex and Lgl antagonizes this activity (Figure 5L).

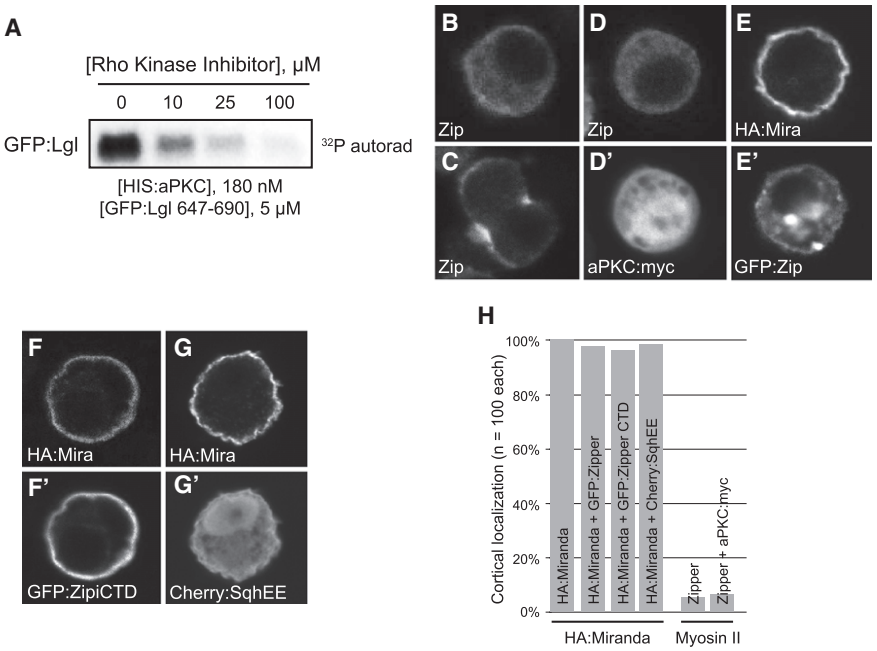


Figure 4. The Rho Kinase Inhibitor Y-27632 Inhibits aPKC, and Myosin II Does Not Affect Miranda Cortical Localization in S2 Cells

(A) aPKC activity is inhibited by the Rho kinase inhibitor Y-27632. An autoradiograph of a GFP fusion of the Lgl phosphorylation site after incubation with aPKC and increasing concentrations of Y-27632 is shown.

(B–H) Expression of the indicated constructs in fixed S2 cells stained by the indicated markers. n = 100 cells for all quantifications.

(B–D) Myosin II is mostly cytoplasmic and enriched at the cleavage furrow in the absence of aPKC:myc (96%) (B and C) and remains cytoplasmic in the presence of aPKC:myc (94%) (D).

(E and F) HA:Miranda remains cortical in the presence of GFP:Zipper (97%) (E) and in the presence of the interphase cortical targeting domain [22] of GFP:Zipper (94%) (F).

(G) Expression of an activated form of *sqh* (Cherry:SqhEE) does not affect cortical HA:Miranda (98%).

(H) Quantification of Miranda and myosin II localization in the presence of the indicated transiently transfected proteins.

Discussion

We have examined the mechanism by which polarity is generated in *Drosophila* neuroblasts, a process required for the segregation of cell fate determinants during asymmetric cell division. This process utilizes aPKC, which is found in many polarized systems such as epithelia. Previously, polarization of the protein Miranda, which is normally restricted to the basal

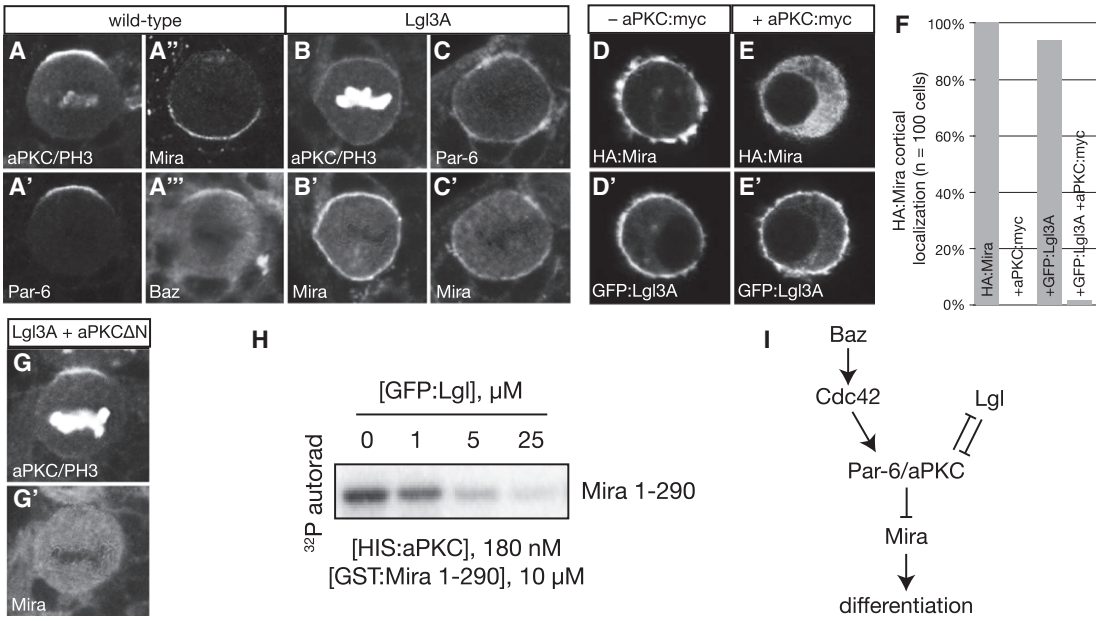


Figure 5. aPKC Can Displace Miranda from the Cortex Independently of Lgl

(A) Wild-type larval (96 hr ALH) brain neuroblasts stained for the indicated markers.

(B) Larval brain neuroblasts expressing Lgl3A (driven by *worniu-GAL4*) exhibit ectopic cortical localization of aPKC (88%; n = 16) and Miranda.

(C) Lgl3A also induces ectopic cortical localization of Par-6 (92%; n = 13).

(D–F) Expression of the indicated constructs in fixed S2 cells stained by the indicated markers. n = 100 cells for all quantifications.

(D and E) Miranda is cortical in the presence of GFP:Lgl3A (94%) in S2 cells (D) but is displaced into the cytoplasm in the presence of GFP:Lgl3A and aPKC:myc (98%) (E).

(F) Quantification of Miranda localization in Lgl3A-expressing background.

(G) Expression of aPKCΔN, which is not efficiently inhibited by Lgl, can displace Miranda from the cortex of neuroblasts even in the presence of Lgl3A (96%; n = 27).

(H) Lgl directly inhibits aPKC. The phosphorylation of GST:Miranda 1–290 by HIS:aPKC as detected by phosphorimaging is reduced upon addition of GFP:Lgl 647–690.

(I) Model of aPKC-induced neuroblast polarity.

neuroblast cortex opposite aPKC, has been thought to occur by a complex cascade of repressive interactions involving the tumor suppressor Lgl and the motor protein myosin II [12, 14]. Our finding that aPKC phosphorylation displaces Miranda from the cortex of neuroblasts and S2 cells led us to suspect that the repressive-cascade model might not accurately describe Miranda displacement. This prompted us to reexamine key results supporting the repressive-cascade model.

Based on our investigation of previous results, we propose that studies suggesting that myosin II is involved in aPKC-mediated cortical displacement of Miranda are an artifact of inhibition of aPKC by the Rho kinase inhibitor Y-27632. Although we cannot exclude the possibility that the Miranda polarity defects observed in Y-27632-treated embryos are indeed the result of myosin II inhibition, the fact that this phenotype is identical to that exhibited by *apkc* mutants, the efficient inhibition of aPKC, and the high concentrations of this compound used in previous reports (~50 mM, compared to the IC_{50} < 10 μM for aPKC and 0.1 μM for Rho kinase) indicate that the simplest interpretation of the Y-27632 phenotype is direct inhibition of aPKC activity. The role of myosin II in Miranda cortical displacement, if any, is unclear.

We have also examined the central result that led to the placement of Lgl between aPKC and Miranda. Expression of a form of Lgl in which the aPKC phosphorylation sites have been inactivated results in uniformly cortical Miranda in neuroblasts [12]. This result can be interpreted in one of two ways: Lgl mediates Miranda cortical targeting and phosphorylation of Lgl represses this activity, or Lgl inhibits aPKC and this inhibition is repressed by aPKC phosphorylation (i.e., feedback). The key distinction between these two models is whether or not aPKC is repressed when Lgl3A is expressed. Several recent studies indicate that Lgl is a potent inhibitor of aPKC activity [5, 23]. Consistent with this, we found that Lgl3A expression dramatically reduces the localization of aPKC to the neuroblast apical cortex. Furthermore, we found that a form of aPKC that is not efficiently repressed by Lgl can overcome the effects of Lgl3A and drive Miranda into the cytoplasm, which is consistent only with Lgl phosphorylation not being a requirement for Miranda cortical displacement. In addition, we showed that Lgl alone is sufficient for inhibition of aPKC activity. Thus, we conclude that Lgl can directly inhibit aPKC and is not required for Miranda cortical targeting.

We favor a simpler mechanism than the repressive-cascade model for Miranda polarization by aPKC: aPKC phosphorylates Miranda, causing it to be displaced from the cortex. The identification of Miranda as a direct aPKC substrate, the requirement of these phosphorylation events for cortical displacement in both S2 cells and neuroblasts, and the necessity of these phosphorylation events for normal development and viability support this model. The sufficiency of phosphorylation (phosphomimetic Miranda is cytoplasmic in the absence of aPKC) indicates that other phosphorylation events (such as phosphorylation of Lgl in the repressive-cascade model) are not required for Miranda cortical displacement. We believe this new model dramatically simplifies our understanding of how asymmetric aPKC activity, as generated by Baz and Cdc42 (Figure 5I), is translated into the segregation of cell fate determinants. Thus, polarization of three components downstream of aPKC, Numb [10], Lgl [12], and Miranda (this work), appears to occur by direct aPKC phosphorylation. Further work will be required to determine whether this mechanism is utilized by all factors that are polarized by aPKC.

Experimental Procedures

S2 Cell Culture and Quantification

S2 cells were cultured in Schneider's medium (Sigma) containing 10% fetal bovine serum. Constructs were cloned into pMT and transfected with Effectene (QIAGEN). We generated alanine and aspartic acid point mutations by site-directed mutagenesis using pMT Miranda as a template. In order to quantify Miranda localization, we analyzed 100 cells transfected with HA:Miranda in the presence or absence of aPKC:myc in ImageJ to generate histograms based on pixel intensity versus pixel distance. We compared the pixel intensity at the cortex to the pixel intensity in the cytoplasm (Figures S1A–S1C). Cells in which the ratio of cortex to cytoplasm staining was 2 or less were scored as cytoplasmic, whereas cells with a ratio greater than 2 were scored as cortical.

Fly Strains

Fly strains used were *Oregon R* (wild-type), lgl^{334} (Bloomington), *worniu-Gal4*, *pros-Gal4*, *UAS-HA:Mira5A*, *UAS-aPKCΔN* [12], *UAS-FLAG:Mira* (gift from C. Doe), *UAS-lgl3A* [12], and $miranda^{ZZ176}$ [16]. Stocks were balanced over *CyO*; *CyO, actin::GFP*; *TM3, actin::GFP, Ser, e*; or *TM3, Sb.* To produce Mira5A transgenic animals, we polymerase chain reaction (PCR) amplified and subcloned the coding sequence into the pUAST vector downstream of a 5′ hemagglutinin (HA) tag and generated transformants via standard methods.

Antibodies and Immunofluorescence Staining

We fixed and stained larval brains and S2 cells as described previously [24]. Wild-type and *lgl* mutant larvae were aged at 25°C until 96 hr after larval hatching (ALH). FLAG:Miranda- and HA:Mira5A-expressing larvae were aged at 30°C until 96 hr ALH (*prospero-Gal4*). HA:Mira5A-expressing embryos were aged at 25°C until stages 11–13. Animals expressing Lgl3A and aPKCΔN were aged at 30°C until 96 hr ALH (*worniu-Gal4*). Rescue experiments were performed by expressing HA:Mira5A (*worniu-Gal4*) in zygotic $miranda^{ZZ176}$ embryos. Primary antibodies used were rabbit anti-PKCζ (C20; 1:1000; Santa Cruz Biotechnology, Inc.), rat anti-Par-6 (1:200) [8], guinea pig anti-Mira (1:500), rat anti-Mira (1:500), rabbit anti-phosphohistone H3 (1:1000; Upstate), guinea pig anti-Baz (1:1000) [25], mouse anti-HA (1:1000; Covance), mouse anti-FLAG (1:100; Sigma), rabbit anti-Zipper (1:2000) [22], rabbit anti-GFP (1:1000; Torrey Pines Biolabs), and mouse anti-Prospero (1:100). Secondary antibodies were from Jackson ImmunoResearch Laboratories and Invitrogen. Confocal images were acquired on a Leica TCS SP2 microscope equipped with a 63×/1.4 NA oil immersion objective. Final figures were arranged with ImageJ, Adobe Photoshop, and Adobe Illustrator.

Protein Purification, Binding Experiments, and Mass Spectrometry

All proteins were expressed and purified as described previously [7]. *Drosophila* embryonic lysates were prepared as described previously [7]. We immunoprecipitated proteins by using ~5 μg rabbit anti-aPKC, rat anti-Par-6, rat anti-Pins [26], or mouse anti-HA with protein G-conjugated beads according to the manufacturer's protocol (GE Healthcare). To determine which immunoprecipitates contained aPKC, we separated samples by SDS-PAGE and transferred them to nitrocellulose, followed by probing with anti-aPKC (1:2000) antibody.

For mass spectrometry, recombinantly purified HIS:Miranda 1–290 was incubated with purified HIS:aPKC as described in the next section. Phosphorylated Miranda 1–290 was purified by SDS-PAGE followed by Coomassie staining. Gel slices containing protein were digested with trypsin and resuspended in formic acid. Samples were analyzed with an LTQ Orbitrap (Thermo Scientific) with neutral loss for +2, +3, and +4 ions. Spectra were analyzed with MASCOT and X!Tandem to identify phosphorylated residues.

Kinase Assay

We incubated HIS:aPKC purified from HEK cells [7] at 30°C for 15 min in reaction buffer (20 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT, 10 mM ATP) and then added GST, GST:Miranda, or HIS:Miranda fragments (10 μM final concentration) and 17 nM [γ-^{32}P]ATP for 20 additional min. We quenched the reaction by addition of SDS loading buffer and heating at 95°C for 5 min. To determine the extent of phosphorylation, we resolved the proteins by SDS-PAGE, exposed the resulting gels to a phosphor screen (Molecular Dynamics), and imaged the screen with a Storm 860.

Supplemental Data

The Supplemental Data include one figure and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)00910-5.

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