Cdc42 acts downstream of Bazooka to regulate neuroblast polarity through Par-6–aPKC

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Summary

Cdc42 recruits Par-6–aPKC to establish cell polarity from worms to mammals. Although Cdc42 is reported to have no function in *Drosophila* neuroblasts, a model for cell polarity and asymmetric cell division, we show that Cdc42 colocalizes with Par-6–aPKC at the apical cortex in a Bazooka-dependent manner, and is required for Par-6–aPKC localization. Loss of Cdc42 disrupts neuroblast polarity: *cdc42* mutant neuroblasts have cytoplasmic Par-6–aPKC, and this phenotype is mimicked by neuroblast-specific expression of a dominant-negative Cdc42 protein or a Par-6 protein that lacks Cdc42-binding ability. Conversely, expression of constitutively active Cdc42 leads to ectopic Par-6–aPKC localization and corresponding cell polarity defects. Bazooka remains apically enriched in *cdc42* mutants. Robust Cdc42 localization requires Par-6, indicating the presence of feedback in this pathway. In addition to regulating Par-6–aPKC localization, Cdc42 increases aPKC activity by relieving Par-6 inhibition. We conclude that Cdc42 regulates aPKC localization and activity downstream of Bazooka, thereby directing neuroblast cell polarity and asymmetric cell division.

Key words: Asymmetric cell division, Cell polarity, Kinase regulation, Par complex

Introduction

Asymmetric cell division is a fundamental mechanism of cellular differentiation. *Drosophila* neural progenitors (neuroblasts) are a model system for studying cell polarity, asymmetric cell division and neural stem cell self-renewal (reviewed in Egger et al., 2007; Yu et al., 2006). *Drosophila* neuroblasts divide unequally to produce a large, apical self-renewing neuroblast and a small, basal ganglion mother cell (GMC) that divides to form two neurons or glial cells. Protein complexes such as Par-6 and atypical protein kinase C (aPKC) are recruited to the neuroblast apical cortex just prior to mitosis, where they direct the polarization of the differentiation factors Miranda (Mira), Prospero (Pros), Brain tumor (Brat), and Numb to the basal cortex (reviewed in Yu et al., 2006). However, the mechanism by which proteins are recruited to the apical cortex is poorly understood.

Par-6 and aPKC are central regulators of neuroblast cell polarity and cell fate. In *par-6* or *aPKC* mutants, the apical protein Bazooka (Baz, also known as Par-3) localizes normally but basal proteins are not excluded from the apical cortex (Petronczki and Knoblich, 2001; Rolls et al., 2003). Thus, Par-6–aPKC is required to restrict Mira, Pros, Brat and Numb differentiation factors to the basal cortex, in part by repressing lethal giant larvae (Lgl), which promotes Mira cortical targeting by antagonizing myosin II function (Barros et al., 2003; Betschinger et al., 2003). In addition to directing neuroblast apical and basal polarity, Par-6–aPKC also regulates neuroblast self-renewal. Reduced aPKC levels lead to depletion of larval neuroblast numbers, whereas misexpression of a membrane-targeted aPKC protein to the basal cortex – but not kinase dead or cytoplasmic proteins – leads to massive expansion of larval neuroblast numbers (Lee et al., 2006b). Thus, precise aPKC localization and activity is essential for proper neuroblast cell polarity, asymmetric cell division and self-renewal.

Despite the importance of Par-6–aPKC localization and activity, very little is known about how Par-6–aPKC localization and activity are regulated in neuroblasts. In many cell types, ranging from worm embryonic blastomeres to mammalian epithelia, the Rho GTPase Cdc42 recruits Par-6–aPKC through direct binding to the Par-6 semi-CRIB domain (Aceto et al., 2006; Joberty et al., 2000; Lin et al., 2000; Schonegg and Hyman, 2006) and induces a conformational change that regulates the activity of its PDZ protein interaction domain (Garrard et al., 2003; Penkert et al., 2004; Peterson et al., 2004). In *Drosophila*, *cdc42* mutants display defects in actin dynamics, intercellular signaling, and epithelial morphogenesis (Genova et al., 2000). Similarly, the interaction between GTP-activated Cdc42 and the Par-6 CRIB domain was shown to be required for the establishment of epithelial polarity in *Drosophila* (Hutterer et al., 2004). However, expression of dominant-negative and constitutively active Cdc42 proteins had no reported effect on embryonic neuroblast cell polarity, despite disrupting epithelial polarity (Hutterer et al., 2004).

Here, we examined the role of Cdc42 in regulating neuroblast polarity and asymmetric cell division using loss of function *cdc42* mutants and neuroblast-specific expression of dominant-negative or constitutively active Cdc42 mutant proteins. We find that Cdc42 is enriched at the apical cortex with Par-6–aPKC in mitotic neuroblasts, and that *cdc42* mutants fail to anchor Par-6–aPKC at the neuroblast apical

cortex, despite the presence of apical Baz protein, leading to severe defects in basal protein localization. Similar phenotypes are observed following neuroblast-specific expression of a dominant-negative Cdc42 protein, or in neuroblasts exclusively expressing a Par-6 protein with CRIB-domain point mutations that abolish Cdc42 binding. In addition, we show that Cdc42 positively regulates aPKC kinase activity by partially relieving Par-6 induced repression. We conclude that Cdc42 plays an essential role in neuroblast cell polarity and asymmetric cell division. Our results open the door for further characterization of Cdc42 regulation and function in neuroblast cell polarity and neural stem cell self-renewal.

Results

Cdc42 is enriched at the apical cortex of asymmetrically dividing neuroblasts

Drosophila Cdc42 has been shown to directly bind Par-6 (Hutterer et al., 2004), so we assayed for Cdc42 colocalization with Par-6 at the apical cortex of mitotic neuroblasts. Antibodies that specifically recognize Cdc42 in tissue are not available, so we expressed a fully functional Cdc42-Myc fusion protein expressed from the native *cdc42* promoter in a *cdc42-3* mutant background (Genova et al., 2000). Mitotic larval neuroblasts show the expected apical cortical crescent of aPKC and Par-6, and we detect Cdc42-Myc enriched at the apical cortex as well as at lower levels around the entire cortex (Fig. 1 and data not shown). Cdc42 remains apically enriched throughout mitosis, paralleling the apical localization of Par-6–aPKC. We conclude that a subset of Cdc42 protein is colocalized with Par-6–aPKC at the apical cortex during neuroblast asymmetric cell division.

Cdc42 acts downstream of Baz to direct Par-6–aPKC localization

A previous study reported no effect on embryonic neuroblast polarity following expression of constitutively active Cdc42 locked in a GTP-bound state (Cdc42V12; hereafter referred to as Cdc42-CA) or dominant-negative Cdc42 locked in a GDP-bound state (Cdc42N17; hereafter referred to as Cdc42-DN) (Hutterer et al., 2004). We repeated these experiments using the same expression system (*pros-Gal4 UAS-cdc42-DN* or *UAS-cdc42-CA*), and confirmed that most mitotic neuroblasts had normal cell polarity (79%, *n*=52). Because the *pros-Gal4* transgene is not expressed in neuroblasts prior to stage 11, after many neuroblasts have divided several times (Pearson and Doe, 2003), we reasoned that using a gal4 line with earlier expression might increase the penetrance of the phenotype. Indeed, when we used *worniu-gal4* – which exhibits neuroblast-specific, high-level expression from the time of neuroblast formation (Albertson and Doe, 2003) – we found a dramatic increase in the percentage of neuroblasts with cell polarity phenotypes. Wild-type embryonic neuroblasts showed normal apical and basal polarity (Fig. 2A), whereas mitotic neuroblasts expressing Cdc42-DN showed expansion of Par-6 and aPKC into the basal cortical domain (79%, *n*=86; Fig. 2B,C), and a corresponding expansion of cortical Mira into the apical cortical domain (45%, *n*=67; Fig. 2B'). The cortical overlap of aPKC and Mira, which is never seen in wild-type neuroblasts, suggests that aPKC is not fully active (see below). Baz showed slightly weaker, but normal, apical localization (100%, *n*=26; Fig. 2D) and division size remained asymmetric

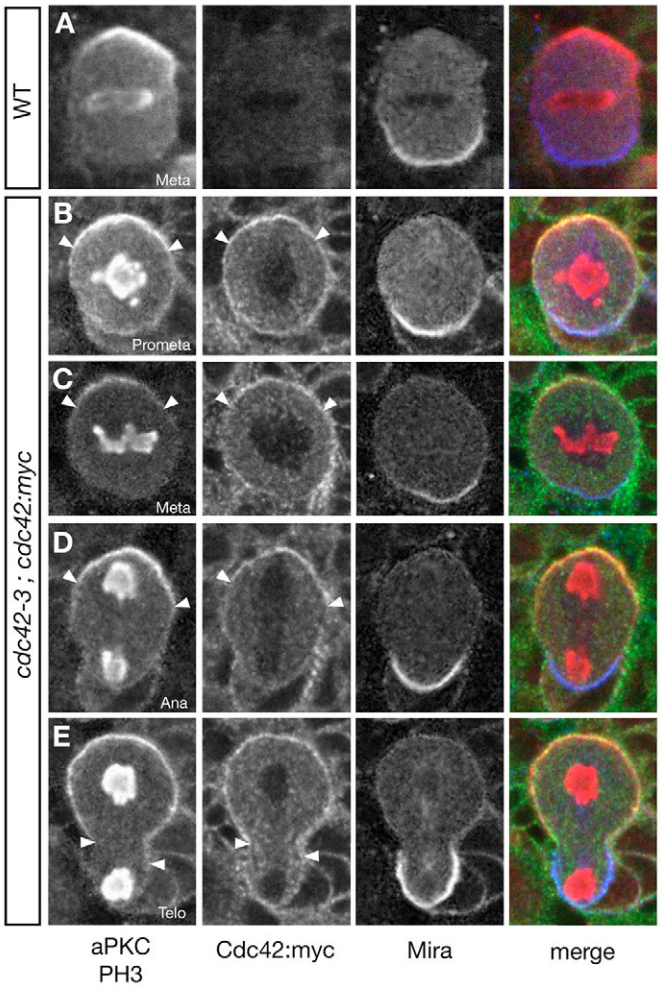

Fig. 1. Cdc42 is enriched at the apical neuroblast cortex. (A) Wild-type central brain neuroblasts 120 hours after larval hatching (ALH). Normal apical and basal protein localization and phosphorylated histone H3 (PH3) are shown with background Myc staining. (B-E) *cdc42-3* central brain neuroblasts at 96 hours ALH expressing Cdc42-Myc under its native promoter (*cdc42-3 ;* cdc42:myc). All stages of mitosis are represented. Arrowheads delineate extent of aPKC (aPKC PH3) and Cdc42-Myc (Cdc42:myc) apical crescents.

(100%, *n*=23; Fig. 2E). We conclude that Cdc42 activity is required downstream of Baz for proper apical localization of Par-6–aPKC.

By contrast, using *worniu-gal4* to drive Cdc42-CA produced uniform cortical Par-6–aPKC with some cytoplamic staining in mitotic neuroblasts (92%, *n*=79; Fig. 2F,G). Delocalization of Mira into the cytoplasm was also observed (94%, *n*=50; Fig. 2F'), consistent with Cdc42 recruitment of active Par-6–aPKC to the entire cortex. No Baz polarity defects were observed, suggesting that Baz cortical localization is Cdc42-independent (100%, *n*=13, Fig. 2I). Importantly, these cell polarity defects were functionally significant, as neuroblast-specific expression of Cdc42-CA produced symmetric divisions in which both neuroblast daughter cells were equal in size (88%, *n*=9; Fig. 2J). We conclude that restricting Cdc42 activity to the apical cortex is essential to establish normal apical Par-6–aPKC localization and subsequent asymmetric cell division.

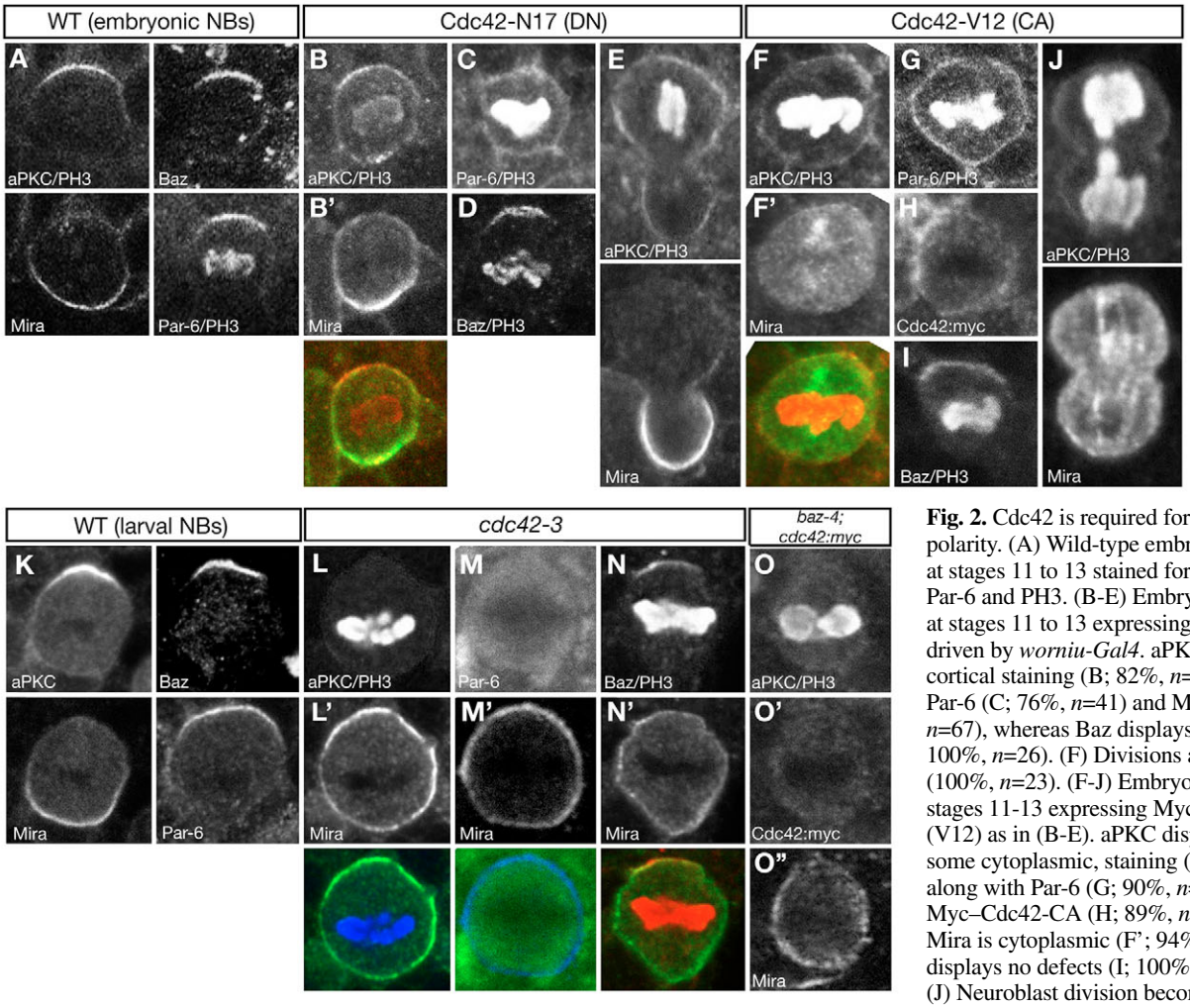

Fig. 2. Cdc42 is required for neuroblast polarity. (A) Wild-type embryonic neuroblasts at stages 11 to 13 stained for aPKC, Baz, Mira, Par-6 and PH3. (B-E) Embryonic neuroblasts at stages 11 to 13 expressing Cdc42-DN (N17) driven by *worniu-Gal4*. aPKC displays ectopic cortical staining (B; 82%, *n*=45) together with Par-6 (C; 76%, *n*=41) and Mira (B'; 45%, *n*=67), whereas Baz displays no defects (D; 100%, *n*=26). (F) Divisions are asymmetric (100%, *n*=23). (F-J) Embryonic neuroblasts stages 11-13 expressing Myc–Cdc42-CA (V12) as in (B-E). aPKC displays cortical, with some cytoplasmic, staining (F; 94%, *n*=50) along with Par-6 (G; 90%, *n*=29) and Myc–Cdc42-CA (H; 89%, *n*=19), whereas Mira is cytoplasmic (F'; 94%, *n*=50). Baz displays no defects (I; 100%, *n*=13). (J) Neuroblast division becomes symmetric upon overexpression of Cdc42-CA (88%, *n*=9). (K) Wild-type central brain neuroblasts 120 hours ALH stained for aPKC, Par-6, Baz, and Mira. (L-N) *cdc42-3* central brain neuroblasts 96 hours ALH. These neuroblasts show cytoplamsic staining of aPKC (L; 84%, *n*=19) and Par-6 (M; 100%, *n*=11), whereas Mira is uniformly cortical (L'-N'; 100%, *n*=46). Baz displays no defects (N; 100%, *n*=16). (O) Cdc42 is mislocalized in zygotic *baz-4* mutant neuroblasts. Embryonic neuroblasts at stages 13 to 14 expressing Cdc42-Myc in a *baz-4* background exhibit loss of Cdc42 apical enrichment. Cdc42-Myc is weakly cortical with some cytoplasmic staining and no apical enrichment (O'), whereas aPKC is cytoplasmic (O) and Mira is uniformally cortical (O"; 100%, *n*=21). (P) Quantification of the Cdc42 requirement for neuroblast polarity in embryonic and larval neuroblasts.

Although both Cdc42-CA and Cdc42-DN generated striking neuroblast cell polarity phenotypes, this could be because of non-specific effects due to the high level of ectopic protein expression. Surprisingly, *cdc42* mutants have never been assayed for neuroblast polarity defects, so we next examined the phenotype of the strong loss-of-function *cdc42-3* allele. *cdc42-3* homozygotes die at late larval stages, but lethality can be rescued by a *cdc42* transgene, showing that the only lethal mutation on the chromosome is *cdc42-3* (Genova et al., 2000). Zygotic *cdc42-3* mutants had normal embryonic and early larval neuroblast polarity (data not shown), presumably because of the large Cdc42 maternal

contribution, so we assayed polarity in third-instar larval central brain neuroblasts. Wild-type larval neuroblasts showed the expected apical crescent of Baz–Par-6–aPKC and basal crescents of Mira (Fig. 2K). By contrast, *cdc42-3* mutant larval neuroblasts showed cytoplasmic Par-6–aPKC (90%, *n*=30; Fig. 2L,M) and uniformly cortical Mira (100%, *n*=46; Fig. 2L'-N'), whereas normal Baz apical crescents were observed (100%, *n*=16; Fig. 2N). Cell-size asymmetry during division could not be assayed because no neuroblasts at telophase were observed, partly because of a substantial decrease in the number of neuroblasts at this late stage of development in these mutants (data not shown). To determine whether Cdc42 acts in parallel or downstream of Baz, we examined Cdc42-Myc localization expressed from the native *cdc42* promoter in zygotic *baz*-mutant embryos. Zygotic *baz* mutant neuroblasts at stage 13 to stage 14 exhibited loss of apical Par-6–aPKC and uniform cortical Mira (data not shown), phenotypes similar to maternal-zygotic *baz*-null germ-line clones (Wodarz et al., 2000). In zygotic *baz*-mutant neuroblasts, Cdc42-Myc showed weak cortical association with no apical enrichment and some cytoplasmic staining in mitotic neuroblasts, whereas aPKC was cytoplasmic and Mira was uniformally cortical (100%, *n*=21; Fig. 2O-O''). Thus, Cdc42 functions downstream of Baz to promote apical cortical localization of Par-6–aPKC.

Cdc42 interaction with Par-6 is required for neuroblast polarity

Although Cdc42 binds Par-6 in *Drosophila* and other organisms, we sought to determine whether Cdc42 functions in neuroblasts through its interaction with Par-6. We first confirmed that the Par-6 CRIB-PDZ domain could bind Cdc42 in vitro (Fig. 3B) and then generated point mutations in conserved residues that abolished this binding (Fig. 3A,B). Mutation of conserved isoleucine and serine to alanines (Par-6^{ISAA}) most effectively eliminated Par-6 CRIB-PDZ binding to Cdc42 (Fig. 3B). To test Par-6^{ISAA} protein for localization and function, we expressed hemagglutinin (HA)-tagged wild-type and Par-6^{ISAA} proteins specifically in neuroblasts lacking endogenous Par-6 protein (Fig. 3C,D). Wild-type HA–Par-6 protein showed normal apical localization in *par6*$^{\Delta 226}$ mutant neuroblasts (Fig. 3C). By contrast, HA–Par-6^{ISAA} protein was cytoplasmic in both wild-type and in *par6*$^{\Delta 226}$ mutants (Fig. 3D; data not shown). Thus, Cdc42–Par-6 binding is required for Par-6 apical cortical localization in neuroblasts. Importantly, the reported Par-6–Baz interaction (Joberty et al., 2000; Lin et al., 2000; Wodarz et al., 2000) is insufficient to target Par-6 to the cortex in the absence of the Cdc42–Par-6 interaction.

We next tested the function of Par-6^{ISAA} in neuroblast polarity. We find that wild-type HA–Par-6 can effectively rescue *par-6* mutants for apical aPKC localization and basal Mira localization (Fig. 3C; data not shown), but that HA–Par-6^{ISAA} shows cytoplasmic aPKC and uniform cortical Mira (Fig. 3D; data not shown). This is identical to the *cdc42-3*-mutant phenotype (Fig. 2). We conclude that Cdc42 binds the Par-6 CRIB-PDZ domain, that this interaction is necessary and sufficient to recruit Par-6 to the neuroblast cortex, and that Cdc42 acts via Par-6 to regulate neuroblast polarity and asymmetric cell division.

Although Baz can localize to the apical cortex independently of Par-6–aPKC (Rolls et al., 2003), Baz is an aPKC substrate (Lin et al., 2000), suggesting that feedback reinforcing apical polarity may exist in this pathway. In this scheme, loss of upstream factors, such as Baz, would abolish apical enrichment (Fig. 2O), whereas loss of downstream factors, such as Par-6 or aPKC, might only reduce Cdc42 apical localization. To test this possibility, we examined Cdc42-Myc localization expressed from the native *cdc42* promoter in zygotic *par6*$^{\Delta 226}$ mutants. Consistent with this model, Cdc42-Myc shows weaker than normal apical localization whereas Mira is uniformly cortical in the absence of Par-6 activity (92%, *n*=12; Fig. 3E; compare with Fig. 1B), indicating that Par-6 is required to maintain normal levels of apically enriched Cdc42.

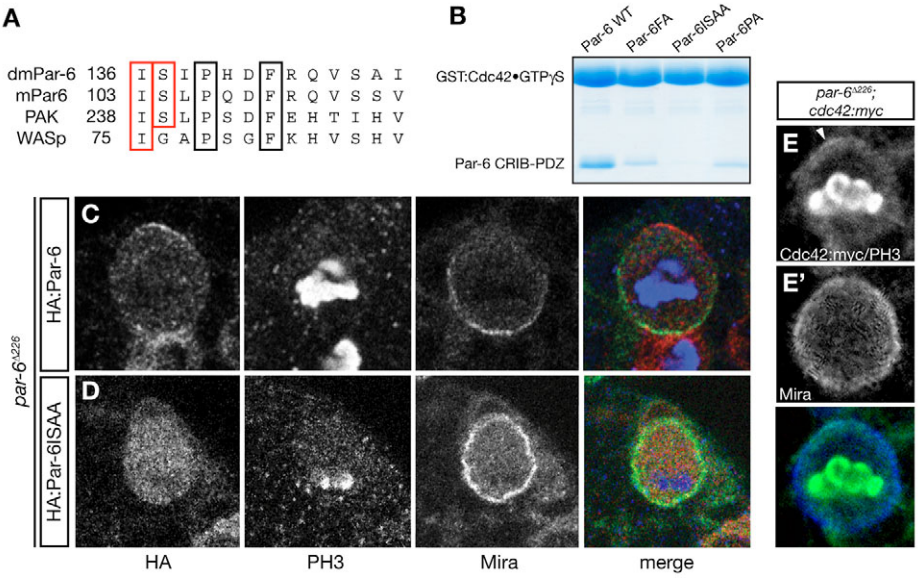

Fig. 3. Cdc42–Par-6 interaction is necessary for neuroblast polarity. (A) Alignment of the Par-6 semi-CRIB domain with CRIB domains from other proteins. Mutated residues are boxed and the residues mutated in the Par-6^{ISAA} transgene are boxed in red. (B) The ISAA mutation disrupts Cdc42 binding to the Par-6 CRIB-PDZ domain. The extent of binding between a glutathione-S-transferase (GST) fusion of [γ^{35}S]GTP-loaded Cdc42 and 55 μM wild-type and mutant Par-6 CRIB-PDZ domains is shown, as determined using a qualitative pull-down assay stained with Coomassie brilliant blue. (C,D) Zygotic *par6*$^{\Delta 226}$ central brain neuroblasts 24 hours ALH expressing *par-6* transgenes. HA–Par-6 (HA:Par-6) localizes to the apical cortex of dividing neuroblasts and rescues Mira phenotype (C). HA–Par-6^{ISAA} (HA:Par-6ISAA) is cytoplasmic and is unable to rescue cortical Mira (D). (E) Zygotic *par6*$^{\Delta 226}$ central brain neuroblasts 24 hours ALH expressing Cdc42-Myc. Arrowhead delineates weak apical enrichment of Cdc42-Myc (92%, *n*=12), whereas Mira is uniformally cortical (100%, *n*=12).

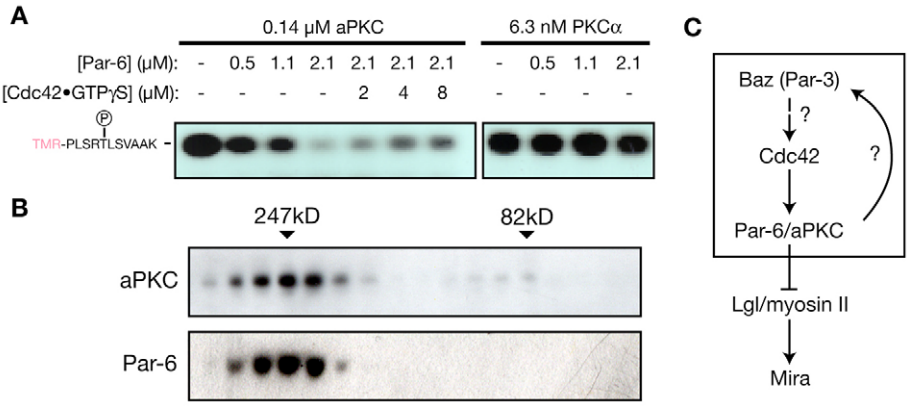

Fig. 4. Par-6 represses whereas Cdc42 partially relieves aPKC kinase activity. (A) Kinase activity of aPKC, Par-6–aPKC, and Cdc42–Par-6–aPKC complexes. The high intrinsic kinase activity of aPKC, expressed and purified from HEK 293 cells, is efficiently repressed by addition of full-length Par-6. Par-6 has no effect on PKCα (right panel). Cdc42 partially restores aPKC activity. The signal is from a rhodamine-labeled peptide corresponding to a PKC consensus substrate (sequence shown on left). (B) aPKC fractionates predominantly with Par-6. Fractions of *Drosophila* embryonic lysate from stages 8 to 14 embryos from a calibrated gel filtration column are shown western blotted with both anti-aPKC and anti-Par-6 antibodies. Very little aPKC fractionates at its native molecular mass (~80 kD) but, instead, co-fractionates with Par-6. (C) Pathway for regulation of apical complex activity in neuroblasts.

Cdc42 relieves Par-6 suppression of aPKC kinase activity

The kinase activity of aPKC displaces Mira from the cortex (Betschinger et al., 2003; Rolls et al., 2003), but expression of Cdc42-DN resulted in aPKC and Mira cortical overlap, suggesting that reduced Cdc42 might regulate aPKC activity. This would be similar to mammals, where Cdc42 activates mammalian PKCλ in a Par-6 dependent manner (Yamanaka et al., 2001), although this has not yet been tested in any other organism. Thus, we tested whether *Drosophila* Cdc42 can activate aPKC in a Par-6 dependent manner. We purified recombinantly expressed *Drosophila* aPKC from HEK 293 cells and measured kinase activity using a fluorescent peptide substrate. As shown in Fig. 4A, aPKC has a high intrinsic activity that is efficiently repressed (approximately five times) by full-length Par-6 (IC_{50} ~450 nM). Par-6 repression of kinase activity is specific to aPKC, because Par-6 had no effect on PKCα activity (Fig. 4A). Addition of Cdc42·[γS]GTP relieves inhibition by Par-6 such that kinase activity is increased approximately twofold over that of the Par-6–aPKC complex. Thus, aPKC has three activation levels: a high intrinsic activity, a very low activity when in complex with Par-6, and an intermediate activity in the ternary Cdc42/Par-6–aPKC complex. To explore whether the high intrinsic activity or the lower activity states of aPKC might be physiologically relevant, we fractionated *Drosophila* embryonic extracts using gel filtration chromatography. Analysis of gel filtration fractions revealed that only a small amount of aPKC fractionates at the molecular weight of aPKC alone (Fig. 4B), suggesting that the high intrinsic activity of aPKC is not a physiologically relevant catalytic state. The partial activation of Par-6–aPKC by Cdc42 might be sufficient to yield proper polarity, or other factors might also activate aPKC at the apical cortex.

Discussion

Little is currently known about how the Par complex is localized or regulated in *Drosophila* neuroblasts, despite the importance of this complex for neuroblast polarity, asymmetric cell division and progenitor self-renewal. Here, we show that Cdc42 plays an essential role in regulating neuroblast cell polarity and asymmetric cell division (Fig. 4C). Baz localizes Cdc42 to the apical cortex where it recruits Par-6–aPKC, leading to polarization of cortical kinase activity that is essential for directing neuroblast cell polarity, asymmetric cell division, and sibling cell fate.

Asymmetric aPKC kinase activity is essential for the restriction of components such as Mira and Numb to the basal cortex (Smith et al., 2007). The aPKC substrates Lgl and Numb are thought to establish basal polarity either by antagonizing activity of myosin II (Barros et al., 2003) or by direct displacement from the cortex (Smith et al., 2007). We have found that Cdc42 recruits Par-6–aPKC to the apical cortex and that Cdc42 relieves Par-6 inhibition of aPKC kinase activity. In the absence of Cdc42, aPKC is delocalized and has reduced activity, resulting in uniform cortical Mira. Expression of Cdc42-DN leads to cortical overlap of inactive Par-6–aPKC and Mira indicating the importance of Cdc42-dependent activation of aPKC kinase activity. Expression of Cdc42-CA leads to cortical aPKC that displaces Mira from the cortex, presumably because Lgl is phosphorylated at the entire cell cortex. This is similar to what is seen when a membrane-targeted aPKC is expressed (Lee et al., 2006b).

Baz, Par-6 and aPKC have been considered to be part of a single complex (the Par complex). We have found that, when Cdc42 function is perturbed, Par-6 and aPKC localization is disrupted but Baz is unaffected. Why is Baz unable to recruit Par-6–aPKC in the absence of Cdc42? One explanation is that Cdc42 modulates the Par-6–Baz interaction, although Cdc42 has no direct effect on Par-6–Baz affinity (Peterson et al., 2004). Alternatively, Baz might only be transiently associated with the Par-6–aPKC complex (e.g. as an enzyme-substrate complex); this is consistent with the observation that Baz does not colocalize with Par-6–aPKC in *Drosophila* embryonic epithelia and its localization is not dependent on either protein (Harris and Peifer, 2005). How does Baz recruit Cdc42 to the apical cortex? Like other Rho GTPases, Cdc42 is lipid modified (prenylated), which is sufficient for cortical localization. Baz is known to bind GDP-exchange factors (GEFs) (Zhang and Macara, 2006), which may induce accumulation of activated Cdc42 at the apical cortex.

The requirement of Par-6 for robust Cdc42 apical enrichment suggests that positive feedback exists in this pathway (Fig. 4C), a signaling pathway property that is also found in polarized neutrophils (Weiner et al., 2002). More work is required to test the role of feedback in neuroblast

polarity but one attractive model is that Baz establishes an initial polarity landmark at the apical cortex in response to external cues (Siegrist and Doe, 2006), which leads to localized Par-6–aPKC activity through Cdc42. Phosphorylation of Baz by aPKC might further increase asymmetric Cdc42 activation, perhaps by increased GEF association, thereby reinforcing cell polarity. Such a mechanism could generate the robust polarity observed in neuroblasts and might explain why expression of dominant Cdc42 mutants late in embryogenesis does not lead to significant defects in polarity (Hutterer et al., 2004).

We are the first to argue that Cdc42 functions downstream of Baz. Cdc42 is required for Baz–Par-6–aPKC localization in *C. elegans* embryos and mammalian neural progenitors (Aceto et al., 2006; Cappello et al., 2006; Kay and Hunter, 2001). In *C. elegans* embryos, RNA interference of *cdc42* disrupts Par-6 localization, whereas PAR-3 localization is slightly perturbed (Aceto et al., 2006; Kay and Hunter, 2001). In this case, Cdc42 is required for the maintenance but not establishment of PAR-3–Par-6 asymmetry (Aceto et al., 2006); however, other proteins have been shown to localize Par complex members independently of Cdc42 (Beers and Kemphues, 2006). Conditional deletion of *cdc42* in the mouse brain causes significant Par-3 localization defects, although this may be caused by the loss of adherens junctions (Cappello et al., 2006). More work will be required in these systems to determine if the pathway that we have proposed is conserved.

We have identified at least two functions of Cdc42 in neuroblasts: first, to recruit Par-6–aPKC to the apical cortex by direct interaction with its CRIB domain and, second, to promote aPKC activity by relieving Par-6 repression. aPKC activity is required to partition Mira and associated differentiation factors into the basal GMC; this ensures maintenance of the apical neuroblast fate as well as the generation of differentiated neurons. Polarized Cdc42 activity may also have a third independent function in promoting physically asymmetric cell division, because uniform cortical localization of active Cdc42 leads to same-size sibling cells. Loss of active Cdc42 at the cortex by overexpression of Cdc42-DN still results in asymmetric cell division, suggesting that other factors also regulate cell-size asymmetry, such as Lgl and Pins (Lee et al., 2006b). In conclusion, our data show that Cdc42 is essential for the establishment of neuroblast cell polarity and asymmetric cell division, and defines its role in recruiting and regulating Par-6–aPKC function. Our findings now allow *Drosophila* neuroblasts to be used as a model system for investigating the regulation and function of Cdc42 in cell polarity, asymmetric cell division and neural stem cell self-renewal.

Materials and Methods

Fly strains

We used Oregon R as a wild-type control. To produce Par-6 wild-type and Par-6 Ile-Ser to Ala-Ala (Par-6^{ISAA}) transgenic animals, we PCR-amplified and subcloned their coding sequences into the pUAST vector downstream of a 5′ hemagglutinin (HA) tag and generated transformants using standard methods. To generate lines expressing HA–Par-6 and HA–Par-6^{ISAA} in a *par-6*-mutant background, we crossed the transgenes with the *worniu-Gal4* driver (Lee et al., 2006a) in a *par6*$^{\Delta 226}$-mutant line. Myc–Cdc42[V12] and Cdc42[N17] (Luo et al., 1994) were expressed in embryonic neuroblasts by crossing lines to *worniu-Gal4* or *pros-Gal4* driver lines at 30°C. Cdc42-Myc was expressed under its native promoter in *cdc42-3*, *par6*$^{\Delta 226}$, and *baz-4* (Bloomington stock 3295) mutant neuroblasts.

Antibodies and immunofluorescent staining

We fixed and stained whole mount embryos and larval brains as previously described (Siegrist and Doe, 2006). Wild-type and *cdc42-3* mutant larvae were aged at 25°C until 96-120 hours after larval hatching (ALH). *par6*$^{\Delta 226}$ mutant larvae were aged at 25°C until 24 hours ALH. All mutant larvae were still responsive to stimuli and no gross degeneration of the cells were observed. Myc–Cdc42[V12] and Cdc42[N17] mutant embryos were aged at 30°C until stages 11 to 13 (*worniu-Gal-4*) or stages 13 to 14 (*pros-Gal4*). *baz-4* mutant embryos were aged at 25°C until stages 13 to 14. Primary antibodies: rabbit anti-PKCζ (C20; 1:1000; Santa Cruz Biotechnology Inc.); rat anti-Par-6 (1:200) (Rolls et al., 2003); guinea pig anti-Mira (1:500); rat anti-Mira (1:500); rabbit anti-phosphorylated histone H3 (1:1000; Upstate); guinea pig anti-Baz (1:1000) (Siller et al., 2006); monoclonal mouse anti-Myc (1:500). 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 using ImageJ, Adobe Photoshop, and Adobe Illustrator.

In vitro binding assay

We produced Par-6 CRIB-PDZ (amino acids 130-255) and Cdc42 proteins as previously described (Peterson et al., 2004). We generated mutated Par-6 (Phe to Ala, Par-6FA; Pro to Ala, Par-6PA; Ile-Ser to to Ala-Ala, Par-6ISAA) by site-directed mutagenesis using pBH Par-6 CRIB-PDZ as a template. All proteins were expressed in the *Escherichia coli* strain BL21 (DE3). Proteins containing His tags were purified on Ni-NTA resin (Qiagen). For GST-pulldown experiments, we adsorbed GST-Cdc42 onto glutathione agarose (Sigma), washed three times with binding buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1% Tween-20), and charged with [γS]GTP as previously described (Peterson et al., 2004). We incubated 55 μM wild-type Par-6 CRIB-PDZ and mutated proteins with GST-Cdc42·[γS]GTP loaded glutathione agarose at room temperature for 15 minutes, and washed five times in binding buffer to remove unbound proteins. To visualize bound proteins, we eluted using SDS sample buffer and analyzed using SDS-PAGE and Coomassie staining. Protein concentrations were determined by Bradford assay using BSA standard controls.

We fractionated *Drosophila* embryonic extracts on a Superdex 200 molecular-sizing column (GE Healthcare) equilibrated in 10 mM HEPES pH 7.5, 100 mM NaCl, and 1 mM DTT and calibrated with a series of molecular weight standards (GE Healthcare). To prepare the lysate, we placed stage 8-14 embryos, dechorinated with 3% bleach (w/v), in embryo lysis buffer [20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and a protease inhibitor cocktail tablet (Roche)], and homogenized with a glass dounce. After two low-speed (18,000 ***g***, 15 minutes) and one high-speed (100,000 ***g***, 30 minutes) centrifugation at 4°C, we injected 100 μl of the resulting supernatant (~10 mg/ml) onto the column and collected 300 μl fractions. To determine which fractions contained Par-6 and aPKC, we separated fractions by SDS-PAGE and transferred to nitrocellulose followed by probing with anti-aPKC (1:2000) or anti-Par-6 (1:1000) antibodies.

Kinase assay

We synthesized a peptide with the sequence PLSRTLSVAAK using FMOC solid-phase synthesis and coupled Rhodamine B (Sigma) as previously described (Qian and Prehoda, 2006). The peptide has a net positive charge that is reduced upon phosphorylation and allows for separation of the two species by agarose gel electrophoresis. We amplified aPKC from an embryonic cDNA library and subcloned it into the mammalian expression vector pCMV containing six histidine codons at the 5′ end. We transfected His-aPKC into FreeStyle™ HEK293 cells (Invitrogen) and collected the cells by centrifugation after 48 hours. We incubated the lysate from these cells with Ni-NTA resin and purified as described above. To measure His-aPKC kinase activity, we incubated the kinase and other factors, as described for Fig. 4, at 30°C for 15 minutes in reaction buffer (100 mM HEPES pH 7.4, 50 mM $MgCl_2$, 5 mM ATP) and then added the fluorescent peptide (50 μM final concentration) for 30 additional minutes. We then quenched the reaction by heating at 95°C for 5 minutes and determined the extent of phosphorylation by gel electrophoresis on 1% agarose in 50 mM Tris-HCl pH 8.0 and visualization on a transilluminator.

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