REGULATION OF CELL POLARITY AND SELF-RENEWAL IN *DROSOPHILA* NEURAL STEM CELLS

by

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A DISSERTATION

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Title: REGULATION OF CELL POLARITY AND SELF-RENEWAL IN *DROSOPHILA* NEURAL STEM CELLS

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The atypical protein kinase C (aPKC) protein has been implicated in several human tumors yet very little is known about how aPKC is regulated. One mechanism that has been proposed as the possible source of several types of tumor is the defective asymmetric cell division of a small number of tumor stem cells. aPKC is required for cell polarization from nematodes to mammals, in tissues as diverse as epithelia, embryonic blastomeres, and neural progenitors. In *Drosophila* central nervous system, mitotic neural stem cells, termed neuroblasts, recruit the polarity proteins aPKC at the cell apical cortex. pack restricts the localization of the differentiation factors Miranda, Prospero, Brat, and Numb to the cell's basal cortex. Later during mitosis, the cytokinetic furrow sets unevenly about the neuroblast apical-basal axis to produce a large cell (neuroblast) which will

continue to divide and self-renew, while the smaller ganglion mother cell inherits differentiation factors and terminally divides to give rise to a pair of neurons and/or glia.

Asymmetric cell division is not only critical for generating cellular diversity, it also ensures that a stable population of neural stem cell is constantly maintained while allowing neurogenesis to occur.

Despite its conserved role in cell polarity and tumorigenesis, relatively little is known about aPKC regulators and targets. In a co-authored work, I show that the small Rho GTPase, Cdc42, indirectly regulates aPKC. However, this stimulation is modest and the mutant phenotypes are not fully penetrant suggesting that other regulators exist.

To isolate other aPKC regulators and targets, I used a biochemical approach to identify aPKC-interacting proteins, and identified one positive regulator and one negative regulator of aPKC. I show that Dynamin-associated protein-160 (Dap160; related to mammalian Intersectin) is a positive regulator of aPKC. I also show that a regulatory subunit of protein phosphatase 2A (PP2A), negatively regulates aPKC.

This dissertation includes both my previously published and my co-authored material.

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I dedicate this work to my parents, thanks for believing in me.

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CHAPTER I

INTRODUCTION TO NEUROBLAST CELL POLARITY AND SELF-RENEWAL

A central question in developmental biology is how a single progenitor can generate multiple cell types. One way of achieving cellular diversity is through asymmetric cell division (ACD). *Drosophila* neural stem cells, termed neuroblasts, are a model system for investigating ACD. Early during embryogenesis, neuroblasts delaminate from the neural ectoderm where they are specified. As the neuroblast enters mitosis, it becomes polarized such that the polarity proteins Par-6 and aPKC are localized to the neuroblast apical cortex. This polarization event will in turn lead to the restriction of the differentiation factors Miranda, Prospero, Brat, and Numb to the neuroblast basal cortex, where they are ultimately partitioned into the smaller, differentiating ganglion mother cell (GMC). The GMC will undergo a terminal division to give rise to a pair of neurons while the neuroblast, which inherits apical proteins, continues to divide and self-renew thus retains neuroblast fate (Caussinus, 2007; Doe, 2008).

ACD is not only critical for generating cellular diversity in *Drosophila* nervous system, but also ensures that an appropriate number of stem cells are constantly maintained while

allowing timely differentiation to occur (Lee et al. 2006a; Lee et al. 2006b). Timely neurogenesis also requires that neuroblasts strictly regulate their cell cycle progression. *Drosophila* neuroblasts are an excellent system to investigate neural stem cell or neuroblasts maintenance and proliferation: neuroblasts born during embryogenesis enter a quiescence period during the embryonic to larval life transition. These neuroblasts will progressively exit quiescence and become proliferative starting early larval life to reach a maximum number of ~100 proliferative neuroblasts per central brain lobe near the end of larval life (Urbach, 2004) thus a change in neuroblast numbers (less than or more than 100 neuroblasts) is suggestive of a defect in neuroblast normal pool size maintenance. Over the years it has become evident that the evolutionary conserved atypical protein kinase C (aPKC) plays a central role in regulating cell polarity and proliferation from nematodes to vertebrates. In mammals, aPKC is required for epithelial polarity establishment and control of cell proliferation. In *Drosophila*, genetic mutations in *aPKC* disrupt the polarity of the basal protein Miranda and cause a reduction in neuroblats.

Atypical protein kinase C proteins are members of the large protein kinase C family. These kinases are ascribed to different sub-families by virtue of their ability to respond to different allosteric stimuli. Classical PKCs (cPKCs) respond to diacyl-glycerol and calcium, novel PKCs (nPKCs) respond to diacyl-glycerol, but atypical PKCs (aPKCs) do not respond to either diacyl-glycerol or calcium and instead are regulated by a poorly understood mechanism. The mammalian aPKC family is composed of two members: *PKC zeta* and *PKC lambda/iota*, which share significant sequence similarity

and are stimulated by insulin and prostaglandins. It is not clear whether *Drosophila* aPKC can be similarly activated.

Despite intensive studies in a variety of systems, how aPKC is regulated is not well understood. The evolutionarily conserved scaffolding protein Par-6 binds aPKC to suppress it activities in mammals (Yamanaka, 2001), however it is not clear whether Par-6 similarly regulates aPKC activity in neuroblasts. Another potential regulator of aPKC is the small G-protein Cdc42. In various systems ranging from worm blastoderms to mammalian epithelia, including *Drosophila* epithelia Par-6 and aPKC localizations require Cdc42 function (Aceto et al., 2006; Joberty et al., 2000; Lin et al., 2000; Schonegg and Hyman, 2006). A role for Cdc42 in regulating Par-6 and aPKC in neuroblasts remains to be established.

In the second chapter, I discuss published co-authored work describing the regulation of aPKC activity by Cdc42. Cdc42 co-localizes with aPKC and can stimulate aPKC activity by relieving Par-6 inhibition of aPKC. Neuroblasts deficient for Cdc42 function show mislocalized aPKC and Par-6 indicating that in addition to regulating aPKC activity, Cdc42 function is required to localize aPKC and Par-6 to the neuroblast apical cortex. However, Cdc42 stimulation of aPKC activity is modest and the polarity phenotypes of *cdc42* mutants are not fully penetrant, which suggests that additional aPKC activators may exist.

In an effort to identify novel aPKC regulators in the context of neuroblast cell polarity and self-renewal, I sought to isolate aPKC-interacting proteins using a biochemical approach, and then assay their function in *Drosophila* neuroblasts. I

performed immunoprecipitation experiments coupled to mass spectrometry analysis (IP/MS) using aPKC as the bait protein and identified Dap160 (dyanamin associated protein-160), a member of the Intersectin protein family (Adams et al., 2000) as a positive aPKC regulator and protein phosphatase 2A (PP2A) as a negative regulator of aPKC. I will discuss Dap160 regulation of aPKC in Chapter III and will devote Chapter V to a discussion on PP2A's role in regulating aPKC. Chapter IV will focus on the functional relationship between Dap160 and the polarity protein Inscuteable (Insc).

Introduction to Inscutable (Insc) polarity in neuroblasts

As neuroblasts delaminate from the embryonic neural ectoderm, Insc the first ever-identified apical protein localize to the apical cortex to regulate the neuroblast mitotic spindle behavior: the neuroblast aligns its mitotic spindle with apical-basal polarity axis. Localization of Insc is dependent on Baz function as *baz* mutant neuroblats fail to recruit Insc to the apical cortex and consequently display spindle orientation defects (Wodarz et al., 1999; Schober et al., 1999). Insc recruits Pins, which in turn recruits Galpha-i (Gαi) to the apical cortex. Loss of Pins results in neuroblast mitotic spindle mis-alignment defects that are similar to what is observed in *insc* mutant neuroblasts (Schaefer et al., 2000; Siegrist and Doe, 2005). The observation that Insc function is required to recruit Pins to the apical cortex and that Insc can directly bind Pins, coupled with the fact that Insc and Pins show similar phenotypes indicate that Insc/Pins interaction is required for proper alignment of the neuroblast mitotic spindle. In the absence of Insc, Pins and Gαi cortical polarity are microtubules-dependent (Siegrist and Doe, 2005), disruption of microtubules in otherwise wild type neuroblasts has no effect on Pins/ Gαi cortical

polarity (Broadus and Doe, 1997; Siegrist and Doe, 2005) thus suggesting that microtubules are parts of a compensatory pathway that regulates cortical polarity in neuroblasts.

Baz and Pins bind Insc at the same 350 amino acids, also Pins has been reported to directly interact with Dlg a members of the tumor suppressor complex. However, it is not clear whether Pins/Insc, Insc/Baz, Pins/Dlg interactions are competitive or synergetic. It is worth mentioning that Baz, Insc, and Pins are interdependent for apical localization in delaminated neuroblasts, suggesting that they are each required for the stability of each one of the above-mentioned interactions.

Insc plays an essential role in the regulation of neuroblast cell polarity and spindle alignment. One important question is how does Insc achieve its apical localization? Are there other cues, in addition to Baz, which regulate Insc localization in neuroblasts? In chapter IV, I present preliminary data suggesting a role for microtubules in regulating Insc polarity in the absence of cortical Baz.

Bridge to Chapter II

In the preceding chapter I underscored the importance of aPKC in the context of cell polarity and self-renewal. I have also indicated that aPKC regulation is poorly understood. Cdc42 function is required for aPKC localization in various systems, however a role for Cdc42 in aPKC regulation in neuroblasts had not been established. In Chapter II, I discuss my previously published and co-authored work revealing a role for Cdc42 in aPKC localization and activity in neuroblasts.

CHAPTER II

CDC42 ACTS DOWNSTREAM OF BAZOOKA TO REGULATE NEUROBLASTS POLARITY THROUGH PAR-6 AND APKC

Atwood, S. X., Chabu, C., Penkert, R. R., Doe, C. Q. and Prehoda, K. E. (2007). J Cell Sci 120, 3200-6.

CONTRIBUTORS:

Scott X. Atwood: Was the main contributor of this manuscript. He produced the majority of the figures for this manuscript and was intimately involved in the design and the analysis of experimental data.

Chiswili Chabu: I provided the preliminary phenotypic characterization of Cdc42 mis-expression experiments in embryonic neuroblasts. More importantly, I analyzed Par-6 transgenes products (wild type and mutant) localization in neuroblasts, tested each transgene's ability to rescue *par-6* polarity phenotypes. These experiments provided an *in vivo* verification of Cdc42 role in regulating aPKC activity via Par-6. Moreover, I was involved in experimental design, data analysis, and manuscript revisions.

Rhiannon R. Penkert: She mapped the minimum residues required for Par-6 binding to Cdc42 in vitro and was instrumental in making the Par-6 (WT) or *Par-6* mutant transgenic fly lines.

Chris Q. Doe and Kenneth E. Prehoda: Principal investigators provided comments and further guidance on experimental design and data analysis.

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 glia. Protein complexes such as Par-6/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; 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/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 via 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 co-localization 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 co-localized 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; called Cdc42-CA below) or dominant negative Cdc42 locked in a GDP-bound state (Cdc42N17; called Cdc42-DN below) (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 use *worniu-gal4* — which exhibits neuroblast-specific, high-level expression from the time of neuroblast formation (Albertson and Doe, 2003) — we find 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

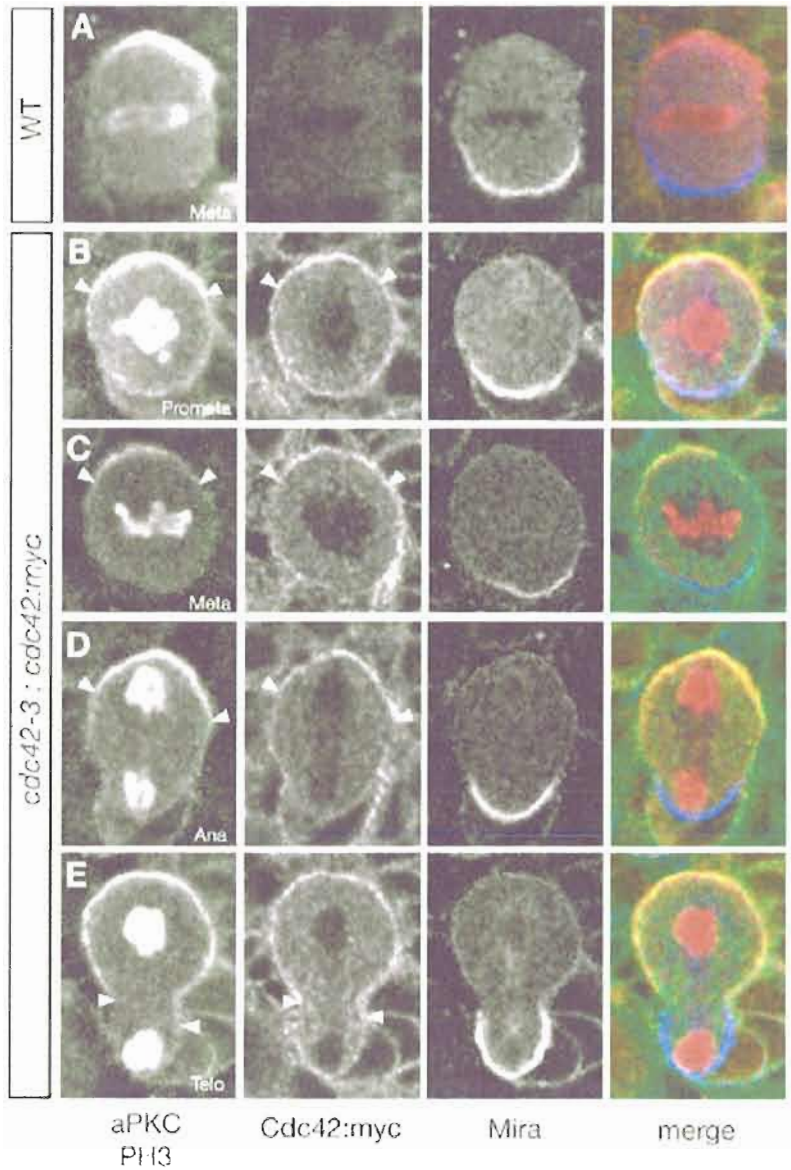


Figure 1. Cdc42 is enriched at the apical neuroblast cortex.

(A) Wild-type central brain neuroblasts at 120 h after larval hatching (ALH). Normal apical and basal protein localization is shown with background c-myc staining.

(B-E) *cdc42-3* central brain neuroblasts at 96 h ALH expressing Cdc42:myc under its native promoter. All stages of mitosis represented. Arrowheads delineate extent of aPKC and Cdc42:myc apical crescents.

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

In 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.

Although both Cdc42-CA and Cdc42-DN generated striking neuroblast cell polarity phenotypes, this could be due to 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). In 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. L'-N'), while normal Baz apical crescents were observed (100%, n=16; Fig. 2N). Cell-size asymmetry during division could not be assayed as no neuroblasts at telophase were observed partly due to 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 stages 13-14 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 uniform cortical (100%, n=21; Fig. 2O-O''). Thus, Cdc42 functions downstream of Baz to promote apical cortical localization of Par-6/aPKC.

Figure 2. Cdc42 is required for neuroblast polarity.

(A) Wild-type embryonic neuroblasts stages 11-13 stained for aPKC, Par-6, Baz, and Mira.

(B-E) Embryonic neuroblasts stages 11-13 expressing Cdc42-DN (N17) driven by *worniu-Gal4*. aPKC displays ectopic cortical staining (B; 82%, n=45) along with Par-6 (C; 76%, n=41) and Mira (B'; 45%, n=67), while Baz displays no defects (D; 100%, n=26). (F) Divisions are asymmetric (100%, n=23).

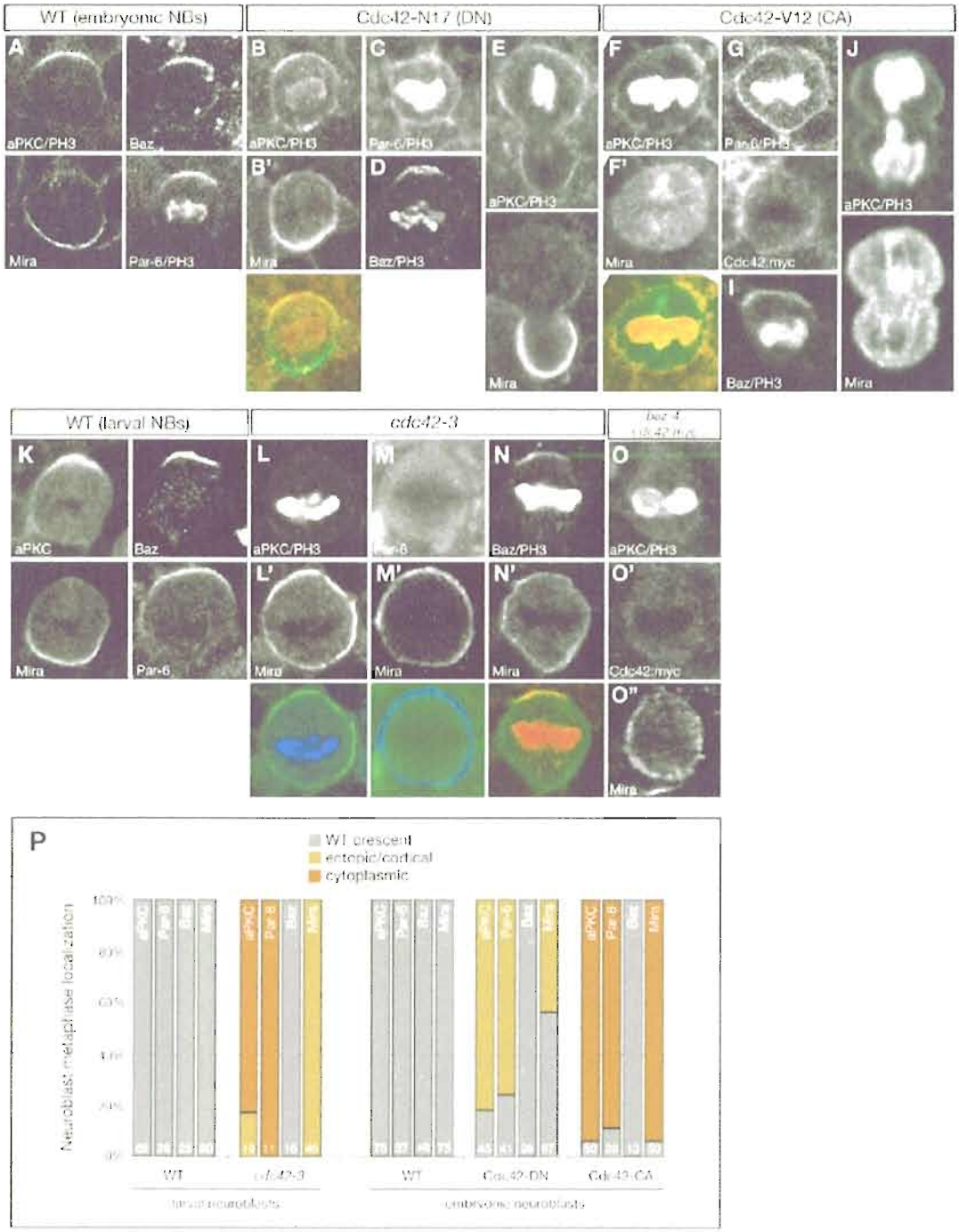
(F-J) Embryonic neuroblasts stages 11-13 expressing myc:Cdc42-CA (V12) as in (B-E). aPKC displays cortical, with some cytoplasmic, staining (F; 94%, n=50) along with Par-6 (G; 90%, n=29) and myc:Cdc42-CA (H; 89%, n=19), while Mira is cytoplasmic (F'; 94%, n=50). Baz displays no defects (I; 100%, n=13). (J) Neuroblast division becomes symmetric upon overexpression of Cdc42-CA (88%, n=9).

(K) Wild-type central brain neuroblasts 120 h ALH stained for aPKC, Par-6, Baz, and Mira.

(L-N) *cdc42-3* central brain neuroblasts 96 h ALH. These neuroblasts show cytoplamsic staining of aPKC (L; 84%, n=19) and Par-6 (M; 100%, n=11), while 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 stages 13-14 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 uniform cortical (O''; 100%, n=21).

(P) Quantification of the Cdc42 requirement for neuroblast polarity in embryonic and larval neuroblasts.


WT (embryonic NBs)
Cdc42-N17 (DN)
Cdc42-V12 (CA)
A
aPKC/PH3
Baz
Mira
Par-6/PH3
B
aPKC/PH3
B'
Mira
C
Par-6/PH3
D
Baz/PH3
E
aPKC/PH3
Mira
F
aPKC/PH3
F'
Mira
G
Par-6/PH3
Cdc42:myc
I
Baz/PH3
J
aPKC/PH3
Mira
WT (larval NBs)
cdc42-3
K
aPKC
Baz
Mira
Par-6
L
aPKC/PH3
L'
Mira
M
Par-6
M'
Mira
N
Baz/PH3
N'
Mira
O
aPKC/PH3
O'
Cdc42:myc
O"
Mira
P
WT crescent
ectopic/cortical
cytoplasmic
Neuroblast metaphase localization
100%
80%
60%
40%
20%
0%
aPKC
Par-6
Baz
Mira
WT
cdc42-3
Cdc42-DN
Cdc42-CA
larval neuroblasts
embryonic neuroblasts

Cdc42 interaction with Par-6 is required for neuroblast polarity

Although Cdc42 binds Par-6 in *Drosophila* and other organisms, we sought to determine if 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). In 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

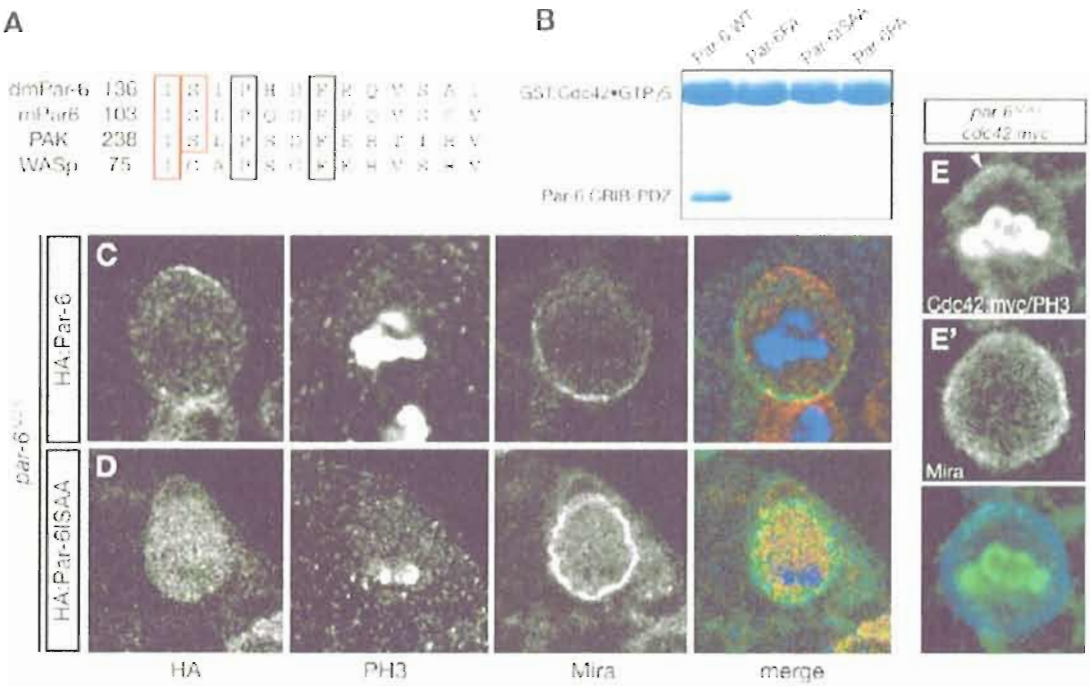


Figure 3. Cdc42/Par-6 interaction is necessary for neuroblast polarity.

(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 GTPγS loaded Cdc42 and 55μM 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 h ALH expressing *par-6* transgenes. HA:Par6 localizes to the apical cortex of dividing neuroblasts and rescues Mira phenotype (C). HA:Par-6^{ISAA} is cytoplasmic and is unable to rescue cortical Mira (D).

(E) Zygotic *par6*$^{\Delta 226}$ central brain neuroblasts 24 h ALH expressing Cdc42:myc. Arrowhead delineates weak apical enrichment of Cdc42:myc (92%, n=12), whereas Mira is uniform cortical (100%, n=12).

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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 independent 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 may 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 to Fig. 1B), indicating that Par-6 is required to maintain normal levels of apically enriched Cdc42.

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-fold) by full-length Par-6 (IC_{50} ~ 450 nM). Par-6 repression of kinase activity is specific to aPKC, as Par-6 had no effect on PKCα activity (Fig. 4A). Addition of Cdc42·GTPγS relieves inhibition by Par-6 such that kinase activity is increased approximately 2-fold 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 reveals 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 may be sufficient to yield proper polarity, or other factors may 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.

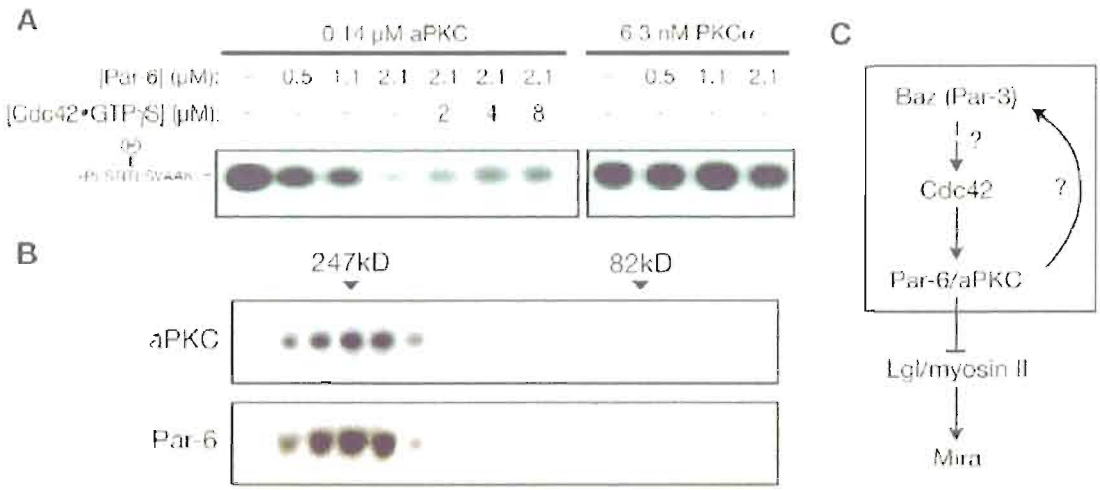


Figure 4. Par-6 represses while Cdc42 partially relieves aPKC kinase activity.

(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 8-14 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 weight (~80kD), but instead co-fractionates with Par-6.

(C) Pathway for regulation of apical complex activity in neuroblasts.

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 Myosin II activity (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 around 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 maybe only 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 (Par-3). 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 *cdc42* RNAi disrupts Par-6 localization, while 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 equal-sized 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 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 h after larval hatching (ALH). *par6*$^{\Delta 226}$ mutant larvae were aged at 25˚C until 24 h 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 stage 11-13 (*worniu-Gal-4*) or stage 13-14 (*pros-Gal4*). *baz-4* mutant embryos were aged at 25˚C until stage 13-14. 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-Phospho-Histone H3 (1:1000; Upstate);

guinea pig anti-Baz (1:1000) (Siller et al., 2006); monoclonal mouse anti-cmyc (1:500). Secondary antibodies were from Jackson ImmunoResearch Laboratories and Invitrogen. Confocal images were acquired on a Leica TCS SP2 microscope equipped with a 63X1.4 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 the Par-6FA, Par-6PA, and 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). 6X HIS-tagged proteins 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 GTPγS as previously described (Peterson et al., 2004). We incubated 55μM wild-type Par-6 CRIB-PDZ and mutated proteins with GST-Cdc42•GTPγS loaded glutathione agarose at room temperature for 15 min, and washed five times in binding buffer to remove unbound proteins. To visualize bound proteins, we eluted with SDS sample buffer and analyzed by SDS–PAGE and Coomassie staining. Protein concentrations were determined by Bradford assay with 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 [20mM HEPES pH 7.5, 100mM NaCl, 1mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, and a protease inhibitor cocktail tablet (Roche)], and homogenized with a glass dounce. After two low-speed (18,000 X g; 15 min) and one high-speed (100,000 X g; 30 min) centrifugation at 4˚C, we injected 100 µl of the resulting supernatant (~10mg/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 a 5' 6X His tag. We transfected His-aPKC into Freestyle HEK 293 cells (Invitrogen) and collected the cells by centrifugation after 48 hrs. We incubating 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 in Fig. 4, at 30˚C for 15 min in reaction buffer (100mM HEPES pH 7.4, 50mM

$MgCl_2$, 5mM ATP) then added the fluorescent peptide (50μM final concentration) for 30 additional min. We then quenched the reaction by heating at 95˚C for 5 min and determined the extent of phosphorylation by gel electrophoresis on 1% agarose in 50mM Tris-HCl pH 8.0 and visualization on a transilluminator.

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

CHAPTER III

DAP160/INTERSECTIN BINDS AND ACTIVATE APKC TO REGULATE CELL POLARITY AND CELL CYCLE PROGRESSION

CONTRIBUTORS:

Chiswili Chabu: my work included designing and conducting all experiments, data analysis, and manuscript preparation.

Chris Q. Doe: Principal investigator provided guidance in experimental design and comments on data analysis. He was instrumental in the organization of the manuscript.

Introduction

Asymmetric cell division has been proposed as a mechanism for maintaining stem cell numbers while allowing the generation of differentiated cell types (Morrison and Kimble, 2006). It is also been proposed that asymmetric cell division of a small number of tumor stem cells may be the origin of several tumor types (Morrison and Kimble, 2006). Asymmetric cell division typically involves establishment of a cell polarity axis, followed by alignment of the mitotic spindle with the polarity axis to produce daughter cells with different molecular composition. One evolutionarily-conserved protein involved in both cell polarity and asymmetric cell division is atypical protein kinase C (aPKC; called PKC-3 in *C. elegans* and aPKC$\lfloor$, $\lfloor/\lceil$ in mammals). aPKC is required for *C. elegans*

blastomere cell polarity (Tabuse et al., 1998), *Drosophila* oocyte polarity (Tian and Deng, 2008); *C. elegans*, *Drosophila* and mammalian epithelial polarity (Aranda et al., 2006; Gopalakrishnan et al., 2007; Harris and Peifer, 2007; Helfrich et al., 2007; Hutterer et al., 2004; Nagai-Tamai et al., 2002; Rolls et al., 2003; Suzuki et al., 2004; Suzuki et al., 2002; Takahama et al., 2008; Yamanaka et al., 2006; Yamanaka et al., 2003; Yamanaka et al., 2001); *Drosophila* planar cell polarity (Djiane et al., 2005); and has been implicated as an oncogene in several human tumors (Fields et al., 2007; Regala et al., 2005; Yi et al., 2008). aPKC is called 'atypical' because unlike classical PKCs or novel PKCs, aPKC is not activated by Ca^{++} or diacylglycerol, but rather is regulated by a relatively poorly understood mechanism of protein-protein interactions.

Drosophila larval brain neural progenitors (neuroblasts) are a powerful system to study the role of asymmetric cell division and stem cell self-renewal and proliferation. aPKC is required for both cell polarity and stem cell self-renewal in *Drosophila* neuroblasts (Lee et al., 2006; Rolls et al., 2003). aPKC co-localizes with the polarity proteins Par-6, Cdc42, and Bazooka (Baz; Par-3 in mammals) at the apical cortex of mitotic neuroblasts, and is segregated into the self-renewing neuroblast at each cell division. The apical cortical crescent of aPKC ensures exclusion of the differentiation factors Miranda, Prospero, Brain tumor (Brat) and Numb from the apical cortex, resulting in their restriction to the basal cortex and ultimate partitioning into the smaller ganglion mother cell (Doe, 2008; Knoblich, 2008), which typically divides once to form two postmitotic neurons. In *aPKC* mutants neuroblasts cell polarity is disturbed such that Miranda and Numb basal proteins become distributed uniformly on the neuroblast cell

cortex, both at metaphase and at telophase, resulting in a molecularly symmetric cell division and a decrease in neuroblast numbers (Lee et al., 2006; Rolls et al., 2003). aPKC has been proposed to regulate neuroblast cortical polarity by phosphorylating and inhibiting cortical localization of the Lethal giant larvae (Lgl) protein (Betschinger et al., 2003), which is required for basal targeting of Miranda and Numb proteins (Ohshiro et al., 2000; Peng et al., 2000). Overexpression of a membrane-targeted aPKC, but not a kinase dead version, leads to reduced basal protein localization and the formation of supernumerary neuroblasts (Lee et al., 2006), revealing the importance of aPKC kinase activity for promoting neuroblast cell polarity and self-renewal. Despite the central role of aPKC kinase activity in establishing neuroblast cell polarity, relatively little is known about how aPKC activity is regulated. Recently we showed that the small G-protein Cdc42 is co-localized with aPKC and can stimulate aPKC activity by relieving Par-6 inhibition of aPKC (Atwood et al., 2007). However, the stimulation of activity is modest and the polarity phenotypes of *cdc42* mutant are not fully penetrant, which suggests that additional aPKC activators may exist.

Despite the importance of aPKC in cell polarity and growth control, little is known about how aPKC activity is regulated. Here we sought to identify aPKC-interacting proteins using a biochemical approach, and then assay their function in Drosophila neuroblasts. I performed immunoprecipitation experiments coupled to mass spectrometry analysis (IP/MS) using aPKC as the bait protein and identified Dap160 (dyanamin associated protein-160), a member of the Intersectin protein family (Adams et al., 2000). Dap160 was originally identified by its ability to interact with the endocytic protein Dynamin in

Drosophila head extracts (Roos and Kelly, 1998). *dap160* mutants have nerve terminals with reduced levels of endocytic proteins (Koh et al., 2004; Marie et al., 2004), consistent with a role in endocytosis. However, *dap160* mutants were also isolated in a genetic screen for modifiers of the Sevenless receptor tyrosine kinase (Roos and Kelly, 1998), showing that Dap160 also regulates signal transduction, similar to mammalian Intersectin (Malacombe et al., 2006; Martin et al., 2006; Tong et al., 2000a; Tong et al., 2000b). Here we show that Dap160 binds aPKC, increases its kinase activity, and that both Dap160 and aPKC are required for neuroblast cell polarity and cell cycle progression.

Materials and methods

Fly stocks and MARCM clones

The wild type fly stock was yellow white (y w). The $dap160^{Q24}$, $Df(2)10523^{\#35}$, and *UAS-Dap160* stocks were gifts from Graeme Davis (UCSF). $dap160^{\Delta 1}$ was a gift from Hugo Bellen (Koh et al., 2004). $aPKC^{k0643}$ flies lacking the early embryonic lethal background mutation were described previously (Rolls et al., 2003). Analysis of cell polarity was carried out in stage 15-16 $dap160^{Q24}/Df(2)10523$ trans-heterozygotes embryos as previously described (Marie et al., 2004). The hypomorphic $dap160^{Q24}$ / $dap160^{\Delta 1}$ hetero-allelic combination was used to assay larval brain neuroblast numbers. The shi^{ts2} chromosome was a gift from Mani Ramaswami (Arizona). To generate positively-marked MARCM clones, we recombined *FRT40* onto the $dap160^{Q24}$ chromosome using standard techniques, and used the previously described *FRTG13* $aPKC^{k06403}$ chromosome (Rolls et al., 2003). *dap160* neuroblast mutant clones were generated by mating $dap160^{Q24}$

FRT40/CyO, actGFP to *y w hsFLP; FRT40A tubP-GAL80/CyO ActGFP; tubP-GAL4 UAS-mCD8::GFP/TM6 Tb Hu* (Bello et al., 2008). *aPKC* ^{k06403} neuroblast mutant clones were generated by mating *FRTG13 aPKC* ^{k06403} */CyO* to *y w hsFLP; FRTG13 tubP-GAL80/CyO actGFP; tubP-GAL4 UAS-mCD8::GFP/TM6 Tb Hu* (Cabernard and Doe unpublished). Clones were induced at 24-28 hours after larval hatching for 1 hour at 37°C and aged for 4 days at 25°C.

In-vitro binding assays and Immuno-precipitations

GST-tagged Dap160 was engineered by polymerase chain reaction from a P-spaceneedle-Dap160-dsred construct (a gift from Graeme Davis) followed by subcloning into pGEX-4T1 vector (Pharmacia). The construct was verified by DNA sequencing. GST-Dap160 was expressed in BL21 cells overnight at 25C, adsorbed 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). We co-incubated GST-Dap160 with Par-6 or aPKC proteins at room temperature for 15 minutes followed by five washes in binding buffer. Interactions were tested by eluting proteins in SDS sample buffer, SDS-PAGE, and western blotting. Par-6 and aPKC proteins were a gift from Scott Atwood (Prehoda lab, Oregon). For immunoprecipitation experiments, a 12 hours collection of y w embryos were homogenized in lysis buffer (50mM HEPES pH7.5, 150mMNaCl, .1% Tween-20, 1 mM EDTA, 2.5 mM EGTA, 10% Glycerol, supplemented with protease inhibitor tablets; Roche) to produce 1 mL of lysate. Lysates were pre-cleared with protein agarose-A beads for 1 hour at 4°C and subsequently divided equally (500 ⌈L each) in two eppendhorf

tubes and incubated with 2 ⌈L of either anti-®gal or anti-aPKC antibodies for 4 hours at 4°C. Lysates were then incubated with protein agarose-A beads for 1 hour at room temperature. For pulldowns, beads were precipitated and washed 3 times in modified lysis buffer containing 1 mM NaCl. Samples were sent to the Fred Hutchinson Cancer Research Center proteomic facility (Seattle,WA) for mass-specrometry analysis.

Kinase activity assays

aPKC activity assays were carried-out as described previously (Atwood et al., 2007). Briefly 0.16 ⌈M aPKC was co-incubated with 50 ⌈M peptide substrate, no Par-6 or 1.14 ⌈M Par-6, no Dap160 or increasing molar concentration of Dap160 protein (0.34, 0.68, 1.02 ⌈M). Phosphorylated peptides were resolved by gel electrophoresis, quantified using ImageJ, and normalized to total peptide input.

Antibodies, immunostaining, and imaging.

An affinity purified anti-Dap160 antibody was raised against a the C-terminal peptide sequence GPF VTS GKP AKA NGT TKK (Alpha Diagnostic). Guinea pig or chicken anti-Dap160 was used at 1:100 (this study); rabbit anti-Dap160, 1:1000 (Bruno Marie, Graeme Davis lab); guinea pig or rat anti-Miranda, 1:500 (Doe lab); rabbit anti-aPKC, 1:1000 (Sigma), Guinea pig anti-Numb, 1:1000 (a gift from Jim Skeath, Missouri); rabbit anti-Scrib, 1:2500 (Doe lab); rat monoclonal anti-Dpn (Doe lab), 1:1; mouse anti-Pros monoclonal (purified MR1A, 1:1000; Doe lab); rabbit anti-phosphohistone H3, 1:1000 (Sigma, St.Louis, MO); rabbit anti-GFP, 1:1000 (Sigma, St.Louis, MO); rat anti-Par-6,

1:250 (Atwood et al., 2007); and mouse anti-® galactosidase, 1:500 (Promega); mouse anti-⟨ tubulin (1:1500, Sigma); guinea pig anti Sec15, 1:1500 (Hugo Bellen); rabbit anti-Rab11, 1-1000 (Donald Ready). Secondary antibodies were obtained from Molecular Probes (Eugene, OR). Embryos were fixed and stained as described previously (Siegrist and Doe, 2005), except that fixing was done in 9% para-formaldehyde for 15 minutes. *shi*^{ts2} embryos were shifted to restrictive temperature (37°C) for 30 minutes, subsequently fixed and stained. Larval brains were dissected, fixed, and stained as described previously (Siller et al., 2005), and analyzed with a Bio-Rad Radiance 2000 or Leica TCS SP laser scanning confocal microscope using a 60x1.4 NA oil immersion objective. Images were processed with Illustrator software (Adobe).

For live imaging we used the GFP protein-trap line G147 *(GFP::Jupiter*) that expresses a microtubule-associated GFP fusion protein (Morin et al., 2001), *worniu-GAL4, UAS-GFP:Miranda, UAS-CHERRY::Jupiter* (Cabernard and Doe unpublished) *and worniu-GAL4; UAS-Dap160/TM6B Tb.* Live imaging was performed as previously described (Siller et al., 2005), except that temporal resolution was either 15 seconds for 2 hours imaging intervals or 3 minutes for overnight imaging sessions. The 4D data sets were processed in ImageJ (NIH) and Imaris (Bitplane, Switzerland) software.

Results

Dap160 and aPKC are part of the same protein complex

To identify novel components in aPKC pathways of neuroblast cell polarity and neuroblast self-renewal we sought to identify aPKC/Par-6 interacting proteins by IP/MS

using aPKC as our bait protein. IP eluates containing complex mixtures of proteins from a non-specific pull-down (anti-green fluorescent protein; GFP) or anti-aPKC pull down were analyzed by Multiple Dimensions Protein Identification Technology (MUDPIT). These experiments returned Dap160 as a potential aPKC-interacting protein with the highest score of tryptic products hits. Dap160 contains two Eps15 homology domains (EH), a coiled-coil domain (CC) and four Src homology 3 domains (SH3) (Fig. 1A). These domains are conserved in the vertebrate Dap160 homologue Intersectin (Fig. 1A), although Dap160 lacks the DH, PH, C2 domains present in the long isoform of Intersectin and consequently is not predicted to have GTPase activity (Fig. 1A). Dap160 has two alternative splice variants: a long isoform, which migrates at 160Kd and a shorter isoform migrating at 120 Kd (Fig. 1A).

Next I sought to verify that Dap160 and aPKC are part of the same protein complex in vivo. An affinity-purified peptide antibody raised against Dap160 C-terminus, which recognizes both Dap160 splice variants of 160 Kd and 120Kd bands in wild type larval lysate but not in dap160 mutant lysate (Fig. 1B), was used to perform aPKC/Dap160 pull down experiments. I found that Dap160 and aPKC reproducibly co-immunoprecipitate (Fig. 1C), thus indicating that Dap160 and aPKC are present in the same protein complex. Similar results were obtained with a second, independently generated Dap160 antibody (Marie et al. 2004) (Supplemental Fig. 1A).

Dap160 directly interacts with aPKC to stimulate aPKC activity

I tested whether Dap160-aPKC directly interact using an in vitro protein interaction assay. We incubated full-length N-terminal GST-tagged Dap160 with full-length N-terminal His-tagged aPKC and found that Dap160 can bind aPKC (Fig. 1D). Par-6 and aPKC form a complex in Drosophila (Atwood et al., 2007), so I tested for direct interactions between Dap160 and Par-6. I found that full-length Dap160 also directly binds Par-6 (Fig. 1E). I conclude that Dap160 directly interacts with both aPKC and Par-6.

Dap160 and aPKC co-localize in neuroblasts

To determine if Dap160 has the potential to activate aPKC in neuroblasts, I examined Dap160 localization during neuroblast asymmetric cell division. In wild type neuroblasts, aPKC is cytoplasmic at interphase but becomes localized to the apical cortex during mitosis (Fig. 2G-K) (Rolls et al., 2003). Similarly, I found that Dap160 protein is cytoplasmic in interphase neuroblasts, but becomes enriched at the apical cortex of mitotic embryonic neuroblasts (Fig. 2A-D). Apical Dap160 protein could not be detected in embryonic *dap160* mutant (Supplemental Fig. 1E) and similar Dap160 localization was observed in wild type embryonic mitotic neuroblasts but in *dap160* mutants using a second independently generated Dap160 antibody (Supplemental Fig. 1B, C) . In mitotic larval neuroblasts, Dap160 was undetectable at the cortex (Fig. 2E), possibly due to low

Figure 1. Dap160 interacts with aPKC

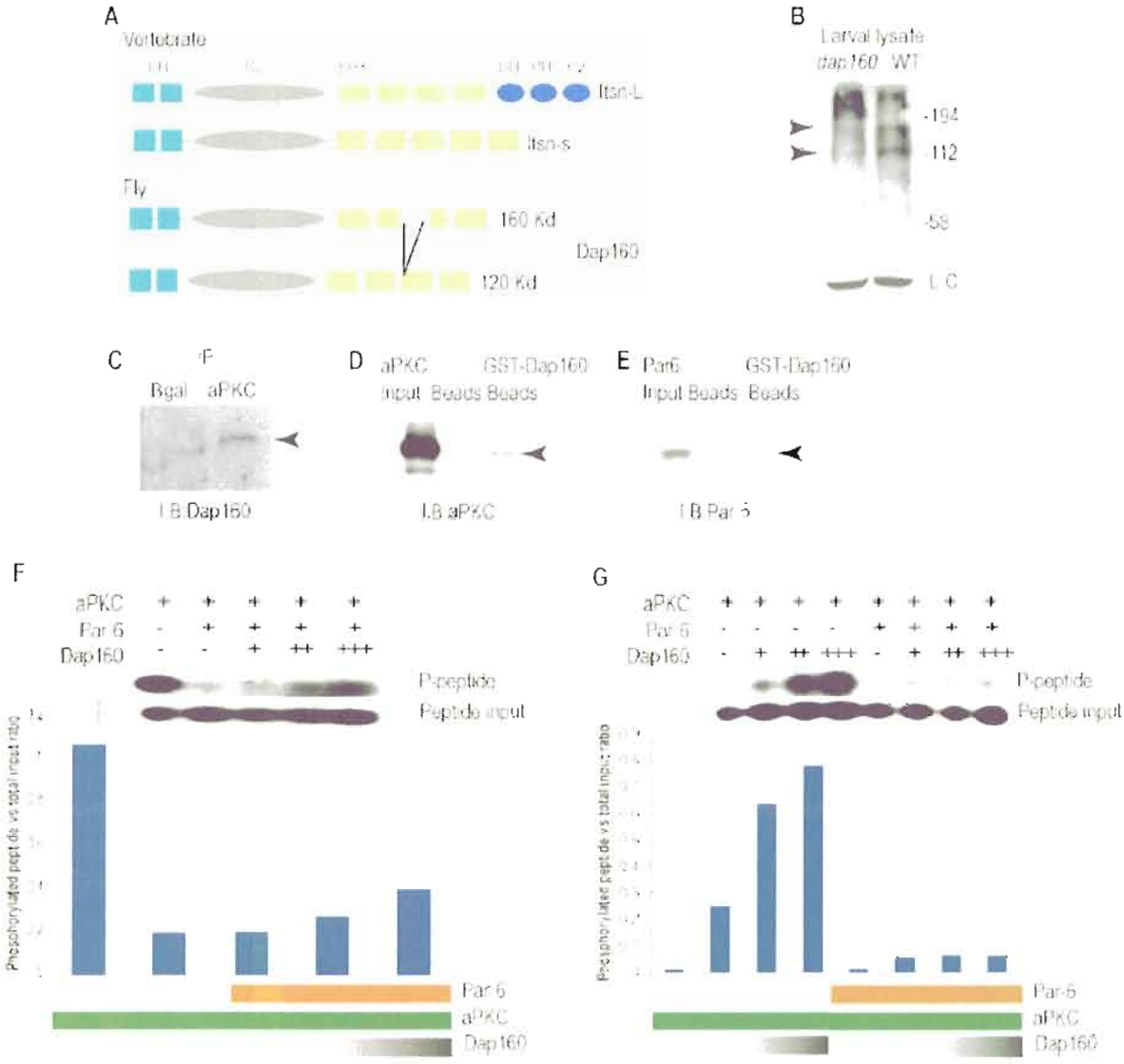
(A) Schematic of protein domains in two vertebrate Intersectin isoforms and the single fly Dap160 isoform.

(B) Western blot showing that Dap160 antibody detects a ~ 160KD band and the 120KD splice variant species of Dap160 (arrowheads) in larval lysate from wild type but not *dap160* mutants.

(C) Immunoprecipitation from larval lysate using an aPKC antibody and a control antibody (Bgal) and blotted with a Dap160 antibody shows aPKC co-immunoprecipitates Dap160 protein (arrowhead).

(D, E) *In-vitro* protein interaction experiments. (D) In vitro generated Dap160 protein coupled to glutathione S-transferase (GST)-beads can bind in vitro produced aPKC protein (arrowheads). Beads alone do not bind aPKC; input lane shown at left. (E) In vitro generated Dap160 protein coupled to glutathione S-transferase (GST)-beads can bind in vitro produced Par-6 protein (arrowheads). Beads alone do not bind Par-6; input lane shown at left.

(F-G) Dap160 directly stimulates aPKC activity, and this effect can be partially blocked by Par6. Top rows depict presence (+) or absence (-) of each protein; middle row shows phosphorylation of the aPKC substrate peptide; and bottom histogram shows quantification of the phosphorylation (bars) over a schematic depiction of protein levels (see methods for protein concentrations). Note that aPKC alone can have high activity immediately after its synthesis (F), or much lower activity after storage (G), but can still be stimulated by Dap160


A
Vertebrate
Itsn-L
Itsn-s
Fly
160 Kd
Dap160
120 Kd
B
Larval lysate
dap160 WT
194
112
58
LC
C
Bgal aPKC
IB Dap160
D
aPKC GST-Dap160
Input Beads Beads
IB aPKC
E
Par6 GST-Dap160
Input Beads Beads
IB Par 6
F
aPKC
Par 6
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P-peptide
Peptide input
Phosphorylated peptide vs total input ratio
Par 6
aPKC
Dap160
G
aPKC
Par 6
Dap160
P-peptide
Peptide input
Phosphorylated peptide vs total input ratio
Par-6
aPKC
Dap160

protein levels and/or reduced antibody detection sensitivity. Therefore, I overexpressed Dap160 in larval neuroblasts to increase its concentration, and in this case we found Dap160 protein enriched at the apical cortex with aPKC in 90% (n=12) of the cells examined (Fig. 2F,L) although weaker ectopic cortical patches were also observed (66%, n=12; Fig. 4K). I conclude that Dap160 and aPKC are cytoplasmic in interphase neuroblasts, and can be co-localized at the apical cortex of mitotic neuroblasts.

Dap160 promotes aPKC apical localization and kinase activity in neuroblasts

Wild type mitotic embryonic neuroblasts have apical aPKC, Par-6, and Baz crescents (Fig. 3A-C) and basal Miranda/Numb cortical crescents (Fig. 3 D-E). We found that in *dap160* mutant embryonic neuroblasts aPKC and Par-6 localization showed ectopic cortical patches beyond the apical cortical domain (71%, n=41 and 50%, n=12 respectively; Fig. 3F,K), while Baz was mostly apical but occasionally cytoplasmic (22%, n=96; Fig. 3G). Cortical aPKC protein may be inactive or less active in *dap160* mutant neuroblasts, because Miranda and Numb proteins showed ectopic cortical localization beyond the basal cortical domain (36%, n=55; Fig. 3I,K and 38%, n=18; Fig. 3J,K), similar to a weak aPKC mutant phenotype (Rolls et al., 2003). *dap160* mutant neuroblasts never showed cytoplasmic Miranda, but rather we observed that aPKC overlapped with Miranda at the cortex (15%, n=13; Fig. 4H,I), which is never observed in wild type neuroblasts (Lee et al., 2006; Rolls et al., 2003). Conversely, Dap160 overexpression in larval neuroblasts results in cortical Dap160 and ectopic cortical patches of aPKC (Fig. 4J,L), as well as depletion of cortical Miranda in some neuroblasts

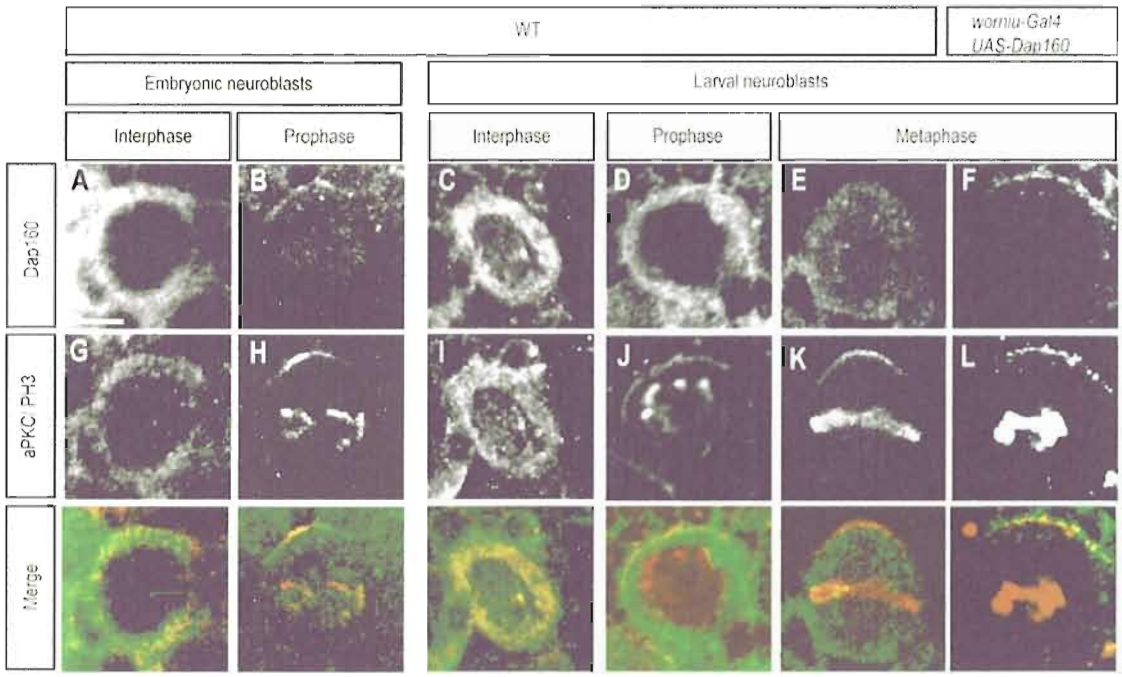


Figure 2. Dap160 co-localizes with aPKC in neuroblasts

Neuroblasts co-stained for Dap160 (A-F) and aPKC (G-L); merged images below. Genotypes, developmental stages, and cell cycle stages labeled at top.

(B,H) Dap160 co-localizes with aPKC at the apical cortex of mitotic embryonic neuroblasts.

(E,K) Endogenous Dap160 is undetectable at the site of aPKC apical cortical localization in larval brain metaphase neuroblasts.

(F,L) Over expression of Dap160 reveals co-localization with aPKC at the apical cortex in larval brain metaphase neuroblasts (90%, n=12). Scale bar is 5 ⌠m in this and all subsequent figures.

(19%, n=21; Fig. 4K), suggesting that the ectopic aPKC is at least partially active.

I further investigated Miranda localization by time-lapse analysis of Dap160-overexpressing larval neuroblasts or wild type neuroblasts expressing *GFP::Miranda* and the microtubule-binding protein *Cherry::Jupiter* (Cabernard and Doe, unpublished). In wild type neuroblasts, GFP::Miranda forms basal crescents during mitosis (100%, n=37; Fig. 4L). In neuroblasts with over expression of Dap160, I could observe divisions where Miranda was cytoplasmic (8%, n=40 divisions from 19 neuroblasts; Fig. 4M), which I never observed in wild type. I conclude that Dap160 positively regulates aPKC localization and activity, and is required for aPKC-mediated neuroblast cortical polarity.

Dap160 regulates the number of proliferating larval neuroblasts

An important function of aPKC is to maintain larval neuroblast pool size: aPKC mutants have fewer proliferating larval brain neuroblasts, and overexpression of a membrane-tethered aPKC increases the number of larval brain neuroblasts (Lee et al., 2006). Here I test whether decreasing or increasing Dap160 levels has a similar effect on brain neuroblast numbers. I find that wild type third instar larval brain lobes contain 96 ± 5 (n=5) Deadpan-positive (Dpn^+) neuroblasts, whereas similarly staged dap160 mutants contain only 72.6 ± 6 (n=5) Dpn^+ neuroblasts (Fig. 4A). I conclude that both Dap160 and aPKC are required to maintain the normal number of proliferating neuroblasts in the larval brain. The loss of neuroblasts in the *dap160* mutant brain could be due to neuroblast death, differentiation, or the failure of neuroblasts to exit from quiescence during larval stages (see Discussion).

To determine if Dap160 role in maintaining neuroblast numbers is lineage-autonomous, I generated *dap160* or *aPKC* mutant single neuroblast clones. I generated single neuroblast clones in first instar larvae, allowed them to develop for 96 hours, and scored them in late third instar larvae (see methods). Without exception, I found that all *dap160* mutant clones contained a single large Dpn^+ neuroblast ($n > 50$; Fig. 4C). Similarly, all *aPKC* single neuroblast mutant clones retained one Dpn^+ neuroblast ($n = 35$; Fig. 4D). The *dap160* mutant clones showed aPKC and Miranda neuroblast polarity phenotypes similar but weaker than observed in zygotic *dap160* mutant embryos (compare Fig. 4H,I to Fig. 3F,I). These results may be due to masking of the *dap160* phenotype by residual Dap160 protein in the mutant clone, or due to a role of Dap160 outside the neuroblast in maintaining neuroblast numbers (see Discussion). Nevertheless, I can conclude that the *dap160* neuroblast cell polarity phenotype is lineage- autonomous.

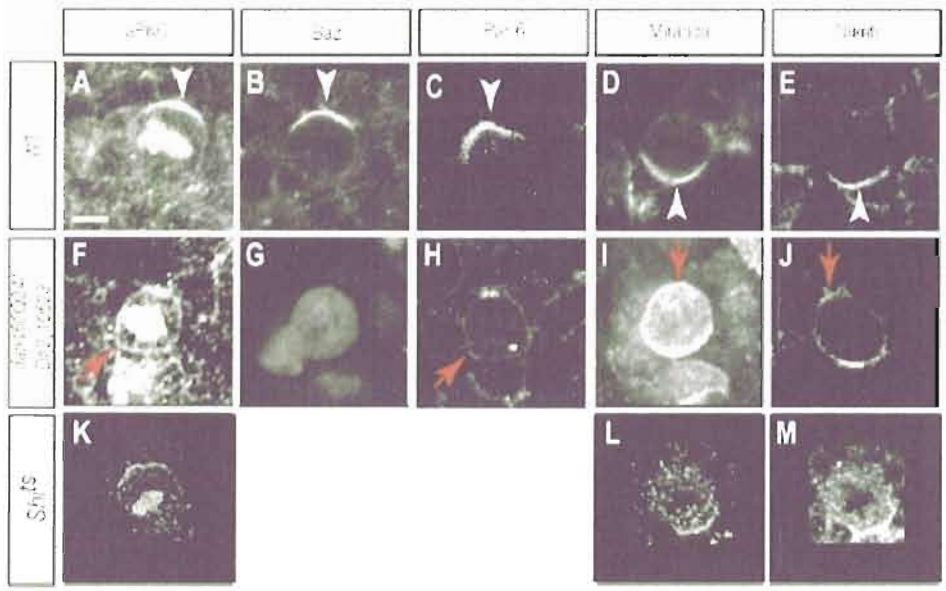
I next determined whether overexpression of Dap160 could affect the number of proliferating neuroblasts. I overexpressed Dap160 using a neuroblast-specific Gal4 driver (*worniu-gal4 UAS-dap160*) and scored the number of Dpn^+ larval neuroblasts. I saw no change in the number of larval neuroblasts at first instar (data not shown). At second larval instar, wild type larvae contain 78 ± 5.6 neuroblasts ($n=4$; Fig. 4A), whereas larvae overexpressing Dap160 have 106.3 ± 13.9 ($n=4$; Fig. 4A). This result is consistent with Dap160 inducing ectopic neuroblast self-renewal, similar but much weaker than the increase in neuroblast numbers seen following overexpression of membrane-tethered aPKC (Lee et al., 2006). At third instar, wild type larvae contain approximately 96 ± 5

Figure 3. Dap160 regulates embryonic neuroblast cortical polarity

(A-E) Wild type metaphase embryonic stage 15 neuroblasts stained for the indicated proteins (top labels) have apical crescents of aPKC, Bazooka, Par-6 (100%, n=23; 100%, n=34; and 96%, n=23 respectively) and basal crescents of Miranda (93%, n=43) and Numb (100%, n=21), (white arrowheads).

(F-J) *dap160* metaphase embryonic stage 15 neuroblasts. aPKC is mostly ectopic cortical aPKC (71%, n=41; F) or cytoplasmic (not shown); Baz is cytoplasmic (21.8%, n=96; G), ectopic cortical or normal (not shown); Par-6 is ectopic cortical (50%, n=12; H, red arrow) or normal (not shown); Miranda is ectopic cortical (red arrow), (36%, n=55; I) or normal (not shown); Numb is ectopic cortical (red arrow) (38%, n=18; J), or normal (not shown).

(K) Quantification of the neuroblast cortical polarity phenotypes. Number of neuroblasts scored is shown as a number at the bottom of each bar.


aPKC
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Miranda
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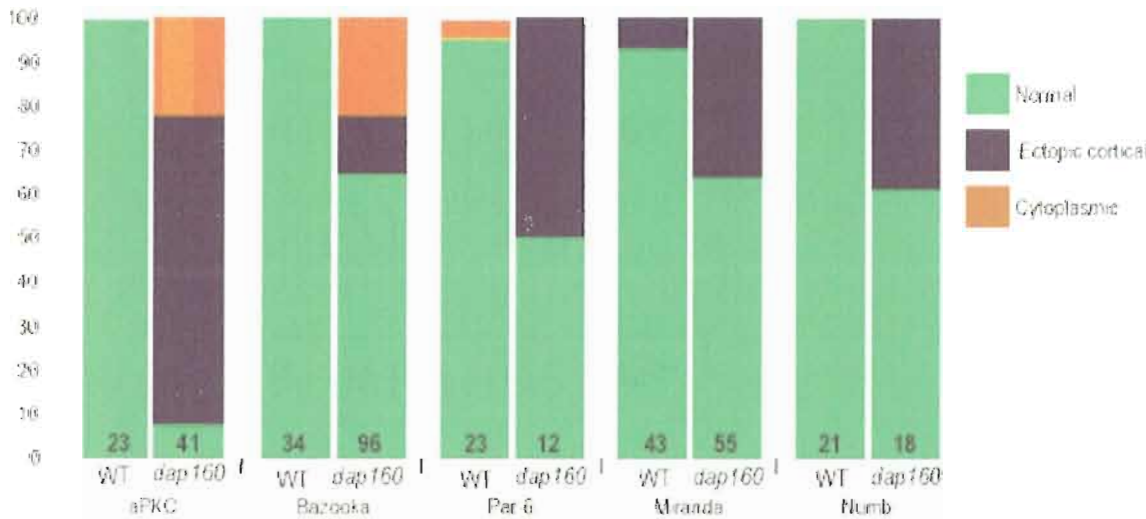
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WT
dap160
aPKC
Bazooka
Par 6
Miranda
Numb
Normal
Ectopic cortical
Cytoplasmic

neuroblasts (n=4; Fig. 4A), whereas larvae overexpressing Dap160 have a decline in neuroblast numbers to ~ 57 ± 10 (n=3; Fig. 4A). This surprising result may be due to neuroblast differentiation, following a graduate accumulation of Miranda/Prospero/Brat differentiation factors in the neuroblasts. This hypothesis is based on my observation that Miranda partitioning into the GMC is defective when assayed in fixed preparations (Figure 4J) or in live imaging of Miranda::GFP localization (Figure 4M). Alternatively, prolonged exposure to high Dap160 levels could lead to neuroblast cell death. Taken together, my mutant and misexpression data support a role for both Dap160 and aPKC in maintaining the number of proliferating neuroblasts in the larval brain.

Dap160 and aPKC are required for neuroblast cell cycle progression

I next tested whether Dap160 and aPKC are required for neuroblast cell cycle progression. I performed time lapse imaging of neuroblast cell cycle progression in both *dap160* and *aPKC* mutant larval neuroblasts expressing the spindle marker GFP::Jupiter. Wild-type neuroblasts take 7.76 ± 2.04 (n=15) minutes to transit from nuclear envelope breakdown (NEBD) to anaphase onset (AO; Fig. 5A; Movie 1), consistent with previous reports (Siller et al., 2006; Siller and Doe, 2008; Siller et al., 2005). In contrast, progression through mitosis (NEBD-AO) was delayed in both dap160 mutants and *aPKC* mutants: 13.37 ± 4.4 minutes, n=10 in *dap160* mutant neuroblasts (Fig. 5B; Movie 2) and 17.84 ± 4.52 minutes, n=11 in *aPKC* mutant neuroblasts (Fig. 5C; Movie 3). In addition, *dap160* mutant neuroblasts had a longer interphase length (~17.5 hours; Movie 4) compared to an average of ~2 hours for wild type neuroblasts (Siller and Doe, 2008; C.

Cabernard and C.Q.D, unpublished results). I conclude that Dap160 and aPKC promote timely cell cycle progression in larval neuroblasts.

Discussion

aPKC plays an important role in regulating cell polarity, neural stem cell identity, and neural stem cell proliferation in vertebrates and invertebrates (Costa et al., 2008; Grifoni et al., 2007; Lee et al., 2006; Rolls et al., 2003). Here I show that Dap160 positively regulates aPKC activity and localization in neuroblasts, and is required for effective aPKC function in establishing neuroblast cell polarity and cell cycle progression.

Dap160 binds aPKC and increases kinase activity

Par-6 directly interacts with aPKC to suppress aPKC activity, while Cdc42 binds Par-6 and modestly upregulates aPKC activity (Atwood et al., 2007; Etienne-Manneville and Hall, 2001; Henrique and Schweisguth, 2003; Hirano et al., 2005). My study shows that Dap160 directly interacts with both aPKC and Par-6, and can stimulate aPKC activity independent of Par-6. However, Par-6 reduces Dap160 ability to stimulate aPKC, suggesting that the Dap160/aPKC complex is more active than Dap160/aPKC/Par6 complex, which in turn is more active than the aPKC/Par6 complex. The exact binding sites for the Dap160/aPKC interaction are unknown; good candidates would be the Dap160 SH3 domains and the two SH3 binding motifs (P-X-X-P) in aPKC.

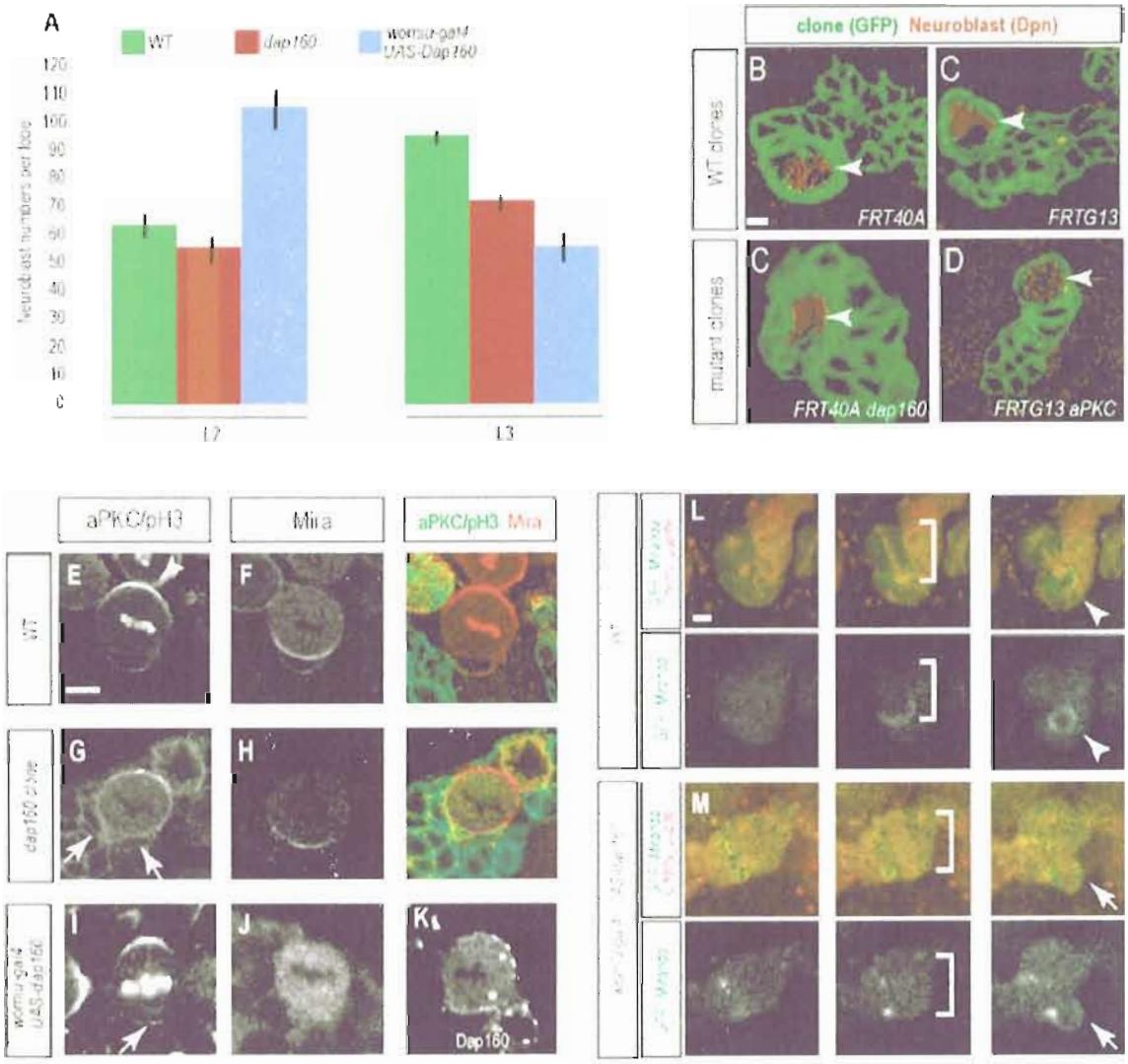
Figure 4. Dap160 positively regulates neuroblast pool size

(A) Neuroblast numbers scored wild type (green bars), *dap160* mutants (red bars), and Dap160 misexpression larvae at second instar (L2) or wandering third instar (L3). See methods for genotypes and growth temperatures.

(B-D) Wild type, *dap160*, and *aPKC* mutant clones ($n \geq 50$ and n=35 respectively) always contain a single Deadpan-positive neuroblast (arrowhead).

(E-K) Neuroblast cortical polarity in larval neuroblasts. (E,F) Wild type neuroblasts have aPKC apical crescents (arrowhead) and Miranda (Mira) basal crescents. (G,H) *dap160* mutants have weak ectopic cortical aPKC (arrows) and normal Mira basal crescents (15%, n=13). (I-K) Dap160 over expression in neuroblasts leads to weak ectopic cortical aPKC (arrow), increased cytoplasmic and reduced cortical Mira (19%, n=21 as shown; remainder normal basal crescents); Dap160 is detected in cortical patches and cytoplasmic puncta (K).

(L, M) Live imaging of wild type neuroblasts with GFP::Miranda and Cherry::Jupiter. (L) Wild type neuroblasts show basal GFP::Mira at metaphase (brackets) and partition GFP::Mira to the GMC at telophase (arrowhead; 100%, n=37). (M) Dap160 misexpressing neuroblasts show cytoplasmic GFP::Mira at metaphase (brackets) and occasionally do not segregate GFP::Mira to the GMC at telophase (arrowhead; 8%, n=40).


A
WT
dap160
worniu-gal4 UAS-Dap160
Neuroblast numbers per lobe
L2
L3
clone (GFP) Neuroblast (Dpn)
WT clones
mutant clones
B
C
C
D
FRT40A
FRTG13
FRT40A dap160
FRTG13 aPKC
aPKC/pH3
Mira
aPKC/pH3 Mira
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dap160 clone
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Dap160
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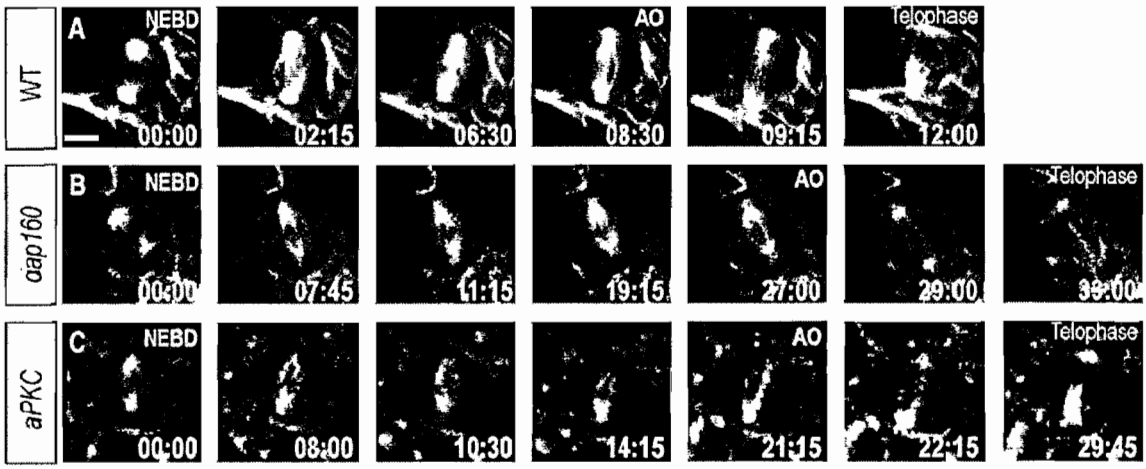


Figure 5. Dap160 is required for neuroblast cell cycle progression

Wild type, *dap160* mutant, and *aPKC* mutant neuroblasts imaged with Jupiter:GFP from nuclear envelope breakdown (NEBD) to anaphase onset (AO). (A) Wild type neuroblasts have a NEBD-AO interval of 7.76 ± 2.04 minutes; n=15. (B) *dap160* mutant neuroblasts have a NEBD-AO interval of 13.37 ± 4.4 minutes; n=10. (C) *aPKC* mutant neuroblasts have a NEBD-AO interval of 17.84 ±4.52 minutes; n=11.

Dap160 regulates aPKC localization

How does Dap160 promote aPKC localization and activity? One possibility is that Dap160 may regulate aPKC localization via its known role in endocytosis, for example by clearing aPKC from the basal cortex. Arguing against this model is my finding that a dynamin mutant (shi^{ts2}), which causes a temperature sensitive inhibition of endocytosis (Ramaswami and Rao, 1993) shifted to the non-permissive temperature showed aPKC, Miranda, and Numb localization patterns clearly distinguishable from *dap160* mutant: although aPKC protein appeared reduced, aPKC localization was restricted to the apical crescent in shi^{ts2} mutant unlike in *dap160* mutant where aPKC cortical localization extended to the basal cortex (compare Fig. 3 A to Fig.3 K). Similarily, Miranda and Numb were unambiguously excluded from the neuroblast apical cortex in shi^{ts2} mutant while they showed ectopic cortical localization in *dap160* mutant, although Miranda and Numb showed an increase in cytoplasmic puncta in shi^{ts2} mutant (compare Fig. 3I to Fig.3L, compare Fig.3J to Fig.3M respectively). I favor a model in which Dap160 regulates aPKC localization via an endocytosis-independent mechanism. In support of endocytosis-independent functions for Dap160, its vertebrate homolog, Intersectin, has both endocytosis and signaling functions. Intersectin regulates endocytosis by serving as a macromolecular scaffold for binding Epsin, Dynamin, and Synaptojanin and localizing to clathrin-coated pits (Hussain et al., 1999); it also has an endocytosis-independent function in binding mammalian Son-of-Sevenless (mSos), recruiting it to the plasma membrane, and thus regulating the Ras signaling pathway (Tong et al., 2000a; Tong et al., 2000b).

Dap160 may promote aPKC localization indirectly, by increasing aPKC kinase activity. aPKC is required to stabilize the Par complex (Baz/Par-3, Par-6, aPKC) in many cell types including neuroblasts (Atwood et al., 2007) and it is likely that lowered aPKC activity would destabilize anchoring proteins such as Bazooka from the neuroblast apical cortex. In support of this model, I observe a weakening of the Bazooka crescent in *dap160* mutant embryonic neuroblasts.

Another possibility is that Dap160 regulates aPKC cortical polarity via vesicle transport. Consistent with this model, Dap160 controls synaptic vesicle transport in Drosophila nerve terminals (Marie et al., 2004), and aPKC (PKC-3), Par-6, and Cdc42 regulate vesicle transport in C. elegans embryos and mammalian cells (Balklava et al., 2007). In fact, Dap160 overexpression in neuroblasts results in enlarged vesicles that are positive for aPKC and the exocyst marker Sec15 (Supplemental Fig. 2 and data not shown), suggesting that Dap160 may direct aPKC-positive vesicles to the apical cortex. One appealing model that awaits testing is that polarized vesicle transport localizes Par proteins to the neuroblast apical cortex, and in turn Par proteins restrict differentiation factors such as Miranda and Numb to the basal cortex.

dap160 mutant embryonic neuroblasts have defects in Baz localization, but *dap160* mutant larval neuroblasts show normal Baz localization (data not shown). This may reflect a difference in the mechanism of Baz localization between embryonic and larval neuroblasts, because *aPKC* mutants also have normal Baz localization in larval neuroblasts (Rolls et al., 2003).

Dap160 and aPKC promote neuroblast cell cycle progression

I provide the first evidence that Dap160 and aPKC promote cell cycle progression in neuroblasts. The related vertebrate aPKCζ regulates cell proliferation in *Xenopus* oocytes, mouse fibroblasts, and in human glioblastoma cell lines (Berra et al., 1993; Donson et al., 2000), indicating that aPKC promotes cell cycle progression in many cell types. Similarly, the vertebrate Dap160-related Intersectin protein is sufficient to induce oncogenic transformation of rodent fibroblasts (Adams et al., 2000), indicating that Intersectin can also promote cell cycle progression. It would be interesting to investigate the relationship between Intersectin and PKCζ in this tumor model system.

Dap160 and aPKC maintain proliferating neuroblast pool size

I find that both *dap160* and *aPKC* mutant larvae have a partial reduction in the number of proliferating neuroblasts (this work; Lee et al., 2006b). This may be due to precocious neuroblast differentiation, because both *dap160* and *aPKC* mutant neuroblasts show delocalization of the basal determinant complex scaffolding protein Miranda into the neuroblast (this work; Rolls et al., 2003). This conclusion is not supported by the finding that both *dap160* and *aPKC* mutant clones always maintain one neuroblast; but I can't rule out the possibility that the clones contain residual aPKC or Dap160 protein that provides enough function to maintain the neuroblast in a proliferative, undifferentiated state. Indeed, neuroblasts in *dap160* mutant clones have a weaker cortical polarity phenotype than neuroblasts in *dap160* organismal mutants, consistent with the presence of residual Dap160 protein in the clones. Alternatively, it is possible that *dap160* mutant

neuroblasts fail to exit from quiescence, leading reduced neuroblast numbers. In this scenario, *dap160* mutant clones would always contain a neuroblast because clones can only be induced in neuroblasts that are already proliferative. However, this seems unlikely due to our finding that *dap160* mutants have normal numbers of neuroblasts during first larval instar. Although it is possible that Dap160 may promote neuroblats exit from quiescence specifically from second-instar to third-instar development. Supporting this notion is the observation that first and second-instar *dap160* mutant brains have wild type numbers of neuroblatsts, however third-instar *dap160* mutant showed reduced neuroblasts numbers compared to wild type. Finally, it is possible that Dap160 acts outside the neuroblast, or outside the CNS, to promote neuroblast maintenance.

Misexpression of Dap160 produces a modest increase in the number of proliferating neuroblasts in second instar larvae, while misexpression of cortically-tethered aPKC results in a dramatic expansion of neuroblast numbers at all larval stages (Lee et al., 2006). It is likely that activity levels of aPKC are limiting in Dap160 overexpression experiments: unknown proteins(s) may oppose Dap160 stimulation of aPKC (similar to Par-6) leading to a weakly active aPKC and thus causing only a modest increase in neuroblats numbers. Surprisingly, we found that prolonged misexpression of Dap160, into the third larval instar, resulted in loss of neuroblasts. This could be due to neuroblast cell death caused by neuroblats continued exposure to elevated levels of Dap160 protein.

Bridge to Chapter IV

In chapter III, I characterize Dap160 function in the context of the polarity of PAR complex proteins. In the following chapter I investigate a role for Dap160 in regulating Inscutable (Insc) cell polarity. A model invoking sequential recruitment of polarity proteins to the neuroblast cell cortex has been proposed in establishment and maintenance of neuroblast cell polarity: PAR complex proteins and Insc are recruited first to the neuroblast apical cortex by an unknown cue. Inscutable in turn recruits Partner of inscutable (Pins) and the small G-protein G alpha-i (G(i) to the neuroblast's apical cortex to align the mitotic spindle with the neuroblast polarity axis (reviewed in Yu et al., 2006). Insc is one of the earliest polarized proteins in neuroblasts and it its function is required for neuroblast cortical polarity as well as for proper neuroblast spindle behavior. However the cues that regulate Insc localization are unknown. In the subsequent chapter, I present preliminary data that suggest that Insc localization is regulated by microtubules in *dap160* mutant neuroblasts.

CHAPTER IV

MICROTUBULES INDUCE INSCUTABLE CORTICAL POLARITY IN *DAP160* MUTANT NEUROBLASTS

CONTRIBUTORS:

Chiswili Chabu: my work included designing and conducting all experiments, data analysis, and manuscript preparation.

Laurina Manning: provided technical assistance for spindle orientation measurements

Chris Q. Doe: Principal investigator provided guidance in experimental design and comments on data analysis.

Background on Insc/Pins cortical polarity and functions in neuroblasts

As the neuroblast enters mitosis, PAR complex proteins (Baz, Par-6, and aPKC) and Inscutable (Insc) localize to the neuroblast apical cortex. Insc localization is followed by a rapid recruitment of Partner of Inscutable (Pins/Rapsynoid) and Galpha-i (G⟨i) to the apical cortex (Cai et al., 2003; Wodarz et al., 1999). The minimal domain for Insc localization has been mapped to a central "asymmetry domain" and the search for this asymmetry domain-interacting proteins lead to the discovery of Pins. While the PAR complex functions to exclude differentiation factors from the apical cortex, the primary function of the Insc/Pins complex is to align the neuroblast's division axis with the

apical-basal axis by stabilizing astral microtubules-apical cortex contacts, although mutations in either Insc or Pins also affect cortical polarity. Through the integration of these two pathways: regulation of cortical polarity and regulation of spindle geometry, neuroblasts are able to differentially partition polarity proteins between itself and the GMC in a highly efficient manner.

Despite its importance in regulating cortical polarity and spindle geometry, little is known about the cues that control Insc localization in neuroblasts. Here I show that Insc cortical polarity in *dap160* mutant neuroblasts is microtubules-dependent.

Materials and methods

Fly stocks

The wild type fly stock was yellow white (y w). For $dap160^{Q24}$ stocks, see above.

Drug treatment and antibodies

To depolymerize microtubules, y w or $dap160^{Q24}$ mutant embryos were treated with 5 ⌈g/ml Colcemid (Sigma) for 1 hour using standard methods (Alberson and Doe, 2003). For immuno-staining we used guinea-pig Baz and rat-⟨tubulin as described above and mouse ⟨tubulin 1:2000, Sigma; rat Pins 1:500, F.Yu; rabbit Insc 1:500, W Chia. Images were acquired and processed as described above.

Results

Dap160 function is required for Pins but not Insc cortical polarity.

Insc cortical polarity requires cortical localization of Baz (Cai et al., 2003; Wodarz et al., 1999), so the observation that a fraction of *dap160* mutant neuroblasts showed cytoplasmic Baz (Fig. 3, Chapter III) prompted me to examine Insc localization in these mutant neuroblasts. I analyzed Insc localization in wild type or *dap160* mutant neuroblasts showing reduced or no cortical baz protein. I found that Insc localization in *dap160* neuroblasts was indistinguishable from wild type despite the reduction or loss of cortical baz (Fig. 1B, J are same cell). To test whether Insc's function to recruit Pins to the neuroblast apical cortex protein is preserved in *dap160* neuroblasts, I examined Pins localization in wild type or *dap160* mutant neuroblasts. Wild type neuroblasts all showed apical Pins (100%, n=20; Fig. 1E) while Pins was apically enriched but showed ectopic localization to the neuroblast's lateral and basal cortex (65%, n=20; Fig. 1F), suggesting that Pins is able to be recruited to the apical cortex but fails to be restricted there (see discussion). I conclude that Insc localization is independent of cortical Baz and that Insc function to recruit Pins to the apical cortex is normal in *dap160* mutant neuroblasts. However, Pins exclusion from the lateral and basal cortical domains requires Dap160 function.

Dap160 function is required for proper division pattern

Early during embryogenesis when neuroblasts delaminate from the neural ectoderm they orient their division axis orthogonal to the overlying epithelium and push GMCs deep

into the tissue, creating a superficial layer comprised of epithelium and neuroblasts and a deeper layer that is populated by GMCs and neurons. During the course of *dap160* mutant analysis, I observed that this organization was lost: GMCs could be seen in the superficial layer intercalated with neuroblasts (data not shown). One possible mechanism that would explain this observation is a randomization of neuroblasts division axis, similar to what is observed in *insc* mutant neuroblasts (Cai et al., 2003; Wodarz et al., 1999). To test a role for Dap160 in regulating neuroblasts division axis or spindle orientation, spindle orientation relative to the epithelium was examined in stage 9-11 Wild type or *dap160* mutant embryos. Wild type neuroblasts aligned their mitotic spindle within 30 degrees range (91%, n=53; Fig. 2A), while spindle orientation was randomized in *dap160* mutant neuroblasts (45%, n=57; Fig. 2B). I conclude that Dap160 function is required for proper spindle orientation in neuroblasts.

Insc cortical polarity in *dap160* mutant neuroblasts is microtubules-dependent

Insc apical crescents always formed above the spindle pole (Fig. 1 D-F) raising the possibility that Insc cortical localization may be induced by microtubules, similar to Pins/G⟨i polarity in *Insc* mutant neuroblasts (Siegrist and Doe, 2005).

Pins/G⟨i microtubules-induced polarity requires the MAGUK family protein Disc-large (Dlg) and the *Drosophila* Kinesin heavy chain 73 (Khc73) (Siegrist and Doe, 2005). Khc 73 function is required for proper alignment of the mitotic spindle relative to the neuroblast apical-basal axis (Siegrist and Doe, 2005). To address the possibility that a similar Dlg/Khc73/microtubules-dependent pathway may regulate Insc cortical polarity

in *dap160* mutant neuroblasts, I first examined Dlg protein localization in wild type or *dap160* mutant neuroblasts and found that *dap160* mutant neuroblasts had normal Dlg localization (Fig. 1 A). Khc73 function is unlikely to be compromised in *dap160* mutant neuroblasts as I did not observe any spindle orientation defects relative to Insc crescents (Fig. 1 D-F).

Finally, to test a role for microtubules in regulating Insc cortical localization in *dap160* mutant neuroblasts, I abolished microtubules in wild type or *dap160* mutant neuroblasts using pharmacological means (see methods). Microtubules were visibly affected by the drug treatment, judging from their morphology. I found that disruption of microtubules had no effect on Insc localization in otherwise wild type neuroblasts (Fig. 2I). In contrast, Insc failed to form apical crescents in *dap160* mutant neuroblasts with compromised microtubules (Fig. 1J). Similarily, Pins formed wild type crescents when microtubules alone were disrupted (Fig. 1M) but Pins was cytoplasmic when both microtubules and Dap160 functions were compromised (Fig. 1N). I conclude that Insc/Pins cortical polarity in *dap160* mutant neuroblasts is microtubules-induced, similar to Pins/G⟨i cortical polarity in the absence of Insc.

Discussion

Insc is expressed in the newly specified neuroblasts and is recruited to the apical cortex by Baz. Insc in turn recruits Pins apically where they function together to regulate neuroblasts spindle behavior. Pins role in regulating spindle behaviour is conserved in

Figure 1. Microtubules are required for Insc cortical polarity in *dap160* mutant neuroblasts

A-C) Dlg and Inscutable localization in *dap160* mutant neuroblats.

(A, C) *dap160* mutant neuroblasts showed normal Disc-large and (B,C) normal Inscutable localization.

D-F) Mitotic spindle alignment with Inscutable crescents in *dap160* mutant neuroblasts.

G,K, and O) Inscutable, Pins, and Bazooka localization in wild type neuroblasts.

(G) Wild type neuroblasts showed apical Insc, (K) apical Pins, and (O) apical Bazooka.

B,H,L,P, and R) Inscutable, Pins, and Bazooka localization in *dap160* mutant neuroblasts.

(B,H) inscutable was normal, (L) Pins was cortical with apical enrichment, (P) Bazooka was cytoplasmic and (R) uniform cortical in *dap160* mutant neuroblasts.

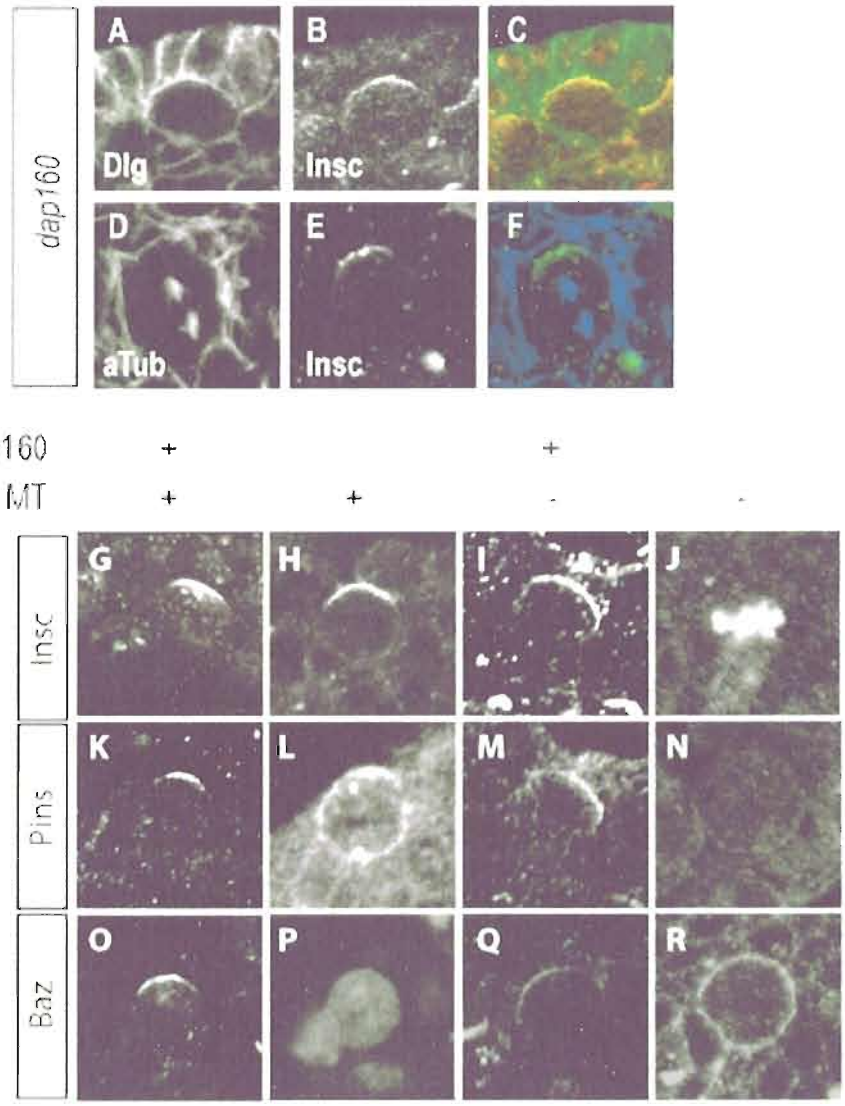
I,M, and Q) Inscutable, Pins, and Bazooka localization in neuroblasts with disrupted microtubules.

(L) Disruption of microtubules in otherwise wild type neuroblasts did not affect Insc, or (M) Pins, or (Q) Bazooka apical localization

J,N) Inscutable, Pins, and Bazooka localization in *dap160* mutant neuroblasts with disrupted microtubules.

(J) Disruption of microtubules in *dap160* mutant neuroblasts caused inscutable to become cytoplasmic.

(N) Similarly, Pins is delocalized in the cytoplasm


dap160
A
Dlg
B
Insc
C
D
aTub
E
Insc
F
Dap160 + +
MT + + - -
Insc
G
H
I
J
Pins
K
L
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Baz
O
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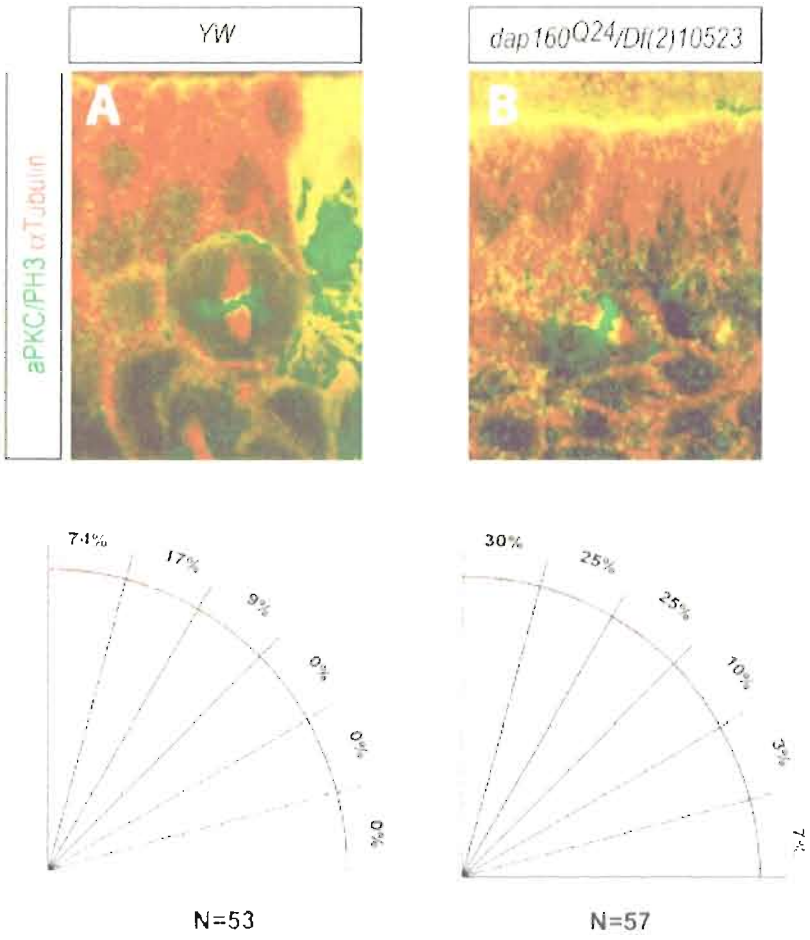


Figure 2. Dap160 function is required for proper division pattern

A-B) Metaphase stage 9-11 wild type or *dap160* mutant embryonic neuroblasts stained with aPKC/PH3 and αTubulin.

A) The majority of wild type neuroblasts aligned their mitotic spindle orthogonally to the overlaying epithelium within 30 degrees (91%, n=52).

B) 45%, n=57 of *dap160* mutant neuroblasts showed randomized spindle orientation.

vertebrates as well as invertebrates. *Insc* and *Pins* mutant neuroblasts exhibit defects in spindle orientation as well as cortical polarity. Yet the cues that govern Insc/Pins localization remains elusive. Here I present preliminary data suggesting that, in the absence of cortical Baz, Insc cortical polarity is induced by microtubules. I also show that Dap160 function is required for restricting Pins at the apical cortex.

Why do Insc and Pins have different localization patterns? I show that Insc forms normal apical crescents in *dap160* mutant neuroblasts but Pins can weakly expand to the basal and lateral domains of the neuroblast's cortex. One possibility in explaining this discrepancy is that microtubules can induce Insc/Pins apical crescents in *dap160* mutant neuroblasts, however in addition of forming apical crescents, Pins is able to less efficiently associate with the neuroblast lateral and basal cortical domains thanks to its ability to bind Dlg, similar to Dlg recruitment of Pins in Sensory Organ Precursors (SOP) progeny P1, where Insc is normally absent (Roegiers et al., 2001). In this model Dap160 would normally preclude Pins-Dlg interaction possibly by sequestering Pins in the cytoplasm. In support of this model, Dlg localization is normal in *dap160* mutant neuroblasts. To test this model, it would be informative to test Pins localization in *dap160, dlg* double mutant neuroblasts.

The *dap160, dlg* double mutants would also be useful in determining whether the microtubules-induced Insc cortical polarity pathway in *dap160* mutant neuroblasts require Dlg function, similar to Pins/Gai cortical polarity in *insc* mutant neuroblasts. The predictions here would be that if Insc apical localization in *dap160* neuroblasts requires

Dlg, then *dap160, dlg* double mutant neuroblasts should be expected to phenocopy *dap160* neuroblasts with compromised microtubules. If on the other end, this pathway is Dlg-independent, then loss of both Dap160 and Dlg will have no effect on Insc localization, however, abolition of microtubules in *dap160, dlg* double mutants should result in the loss of apical Insc. The latter outcome would indicate that Insc cortical polarity in *dap160* mutant neuroblasts is induced by a novel Dlg-independent, microtubules-regulated pathway, which would open up the doors for future research.

How does Dap160 regulate neuroblasts spindle orientation? One hypothesis is that Dap160 functions via aPKC. PAR complex activity, specifically Bazooka, is required for normal spindle orientation in neuroblasts (Cai et al., 2003; Wodarz et al., 1999). In Chapter III, I show that aPKC activity is lost/reduced and that Baz/Par-6 cortical polarity are affected in *dap160* mutant neuroblasts, thus spindle orientation defects observed in *dap160* mutant neuroblasts are likely attributable to loss of/reduced aPKC activity.

To summarize my observations in this chapter and in chapter III, I propose a model where Dap160 functions directly downstream of Insc but upstream of PAR proteins in regulating neuroblasts cortical polarity. The following observations support this model: first, *dap160* mutant neuroblasts show normal Insc but abnormal PAR proteins and Miranda localizations. Second, these localization defects are qualitatively similar to defects observed in *insc* mutant neuroblasts. Finally, *dap160* and *insc* mutant neuroblasts have similar spindle orientation defects (this study; Cai et al., 2003). In this model, Dap160 and microtubules would be part of a redundant pathway that regulates Insc cortical polarity. Consistent with this hypothesis, disruption of microtubules in

otherwise wild type neuroblats has no effect on Insc localization and neither does the loss of Dap160 function. However, disruption of both Dap160 and microtubules functions results in the failure of Insc to form apical crescent.

Bridge to chapter V

In chapter III, I described how I became interested in Dap160 and I subsequently characterize Dap160 as a positive regulator of aPKC, thus a regulator of neuroblast cortical polarity. In the following chapter I focus on protein phosphatase 2A(PP2A) and discuss its role as a negative regulator of aPKC.

CHAPTER V

TWINS, A SUBUNIT OF THE PROTEIN PHOSPHATASE 2A (PP2A), REGULATES NEURAL PROGENITOR CELL FATE VIA APKC

CONTRIBUTORS:

Chiswili Chabu: my work included designing and conducting all experiments, data analysis, and manuscript preparation.

Chris Q. Doe: Principal investigator provided guidance in experimental design and comments on data analysis.

Introduction

Phosphorylation of proteins regulated by kinases is widely used in eukaryotes as a means to regulate cellular processes. These phosophorylation events are also controlled by phosphatase, which dephosphorylates kinases and/or kinases substrates thereby regulating their cellular activity. Several *Drosophila* kinases, including aPKC, have been implicated in the regulation of neuroblast cell polarity and self-renewal (reviewed in chia 08) however very little is known about the regulation of these kinases and/or their substrate(s). In an effort to identify aPKC-interacting protein thus potential aPKC

regulators, I performed a biochemical screen (see chapter III) and identified Protein phosphatase 2A (PP2A) as a potential regulator of aPKC. PP2A is a Serine/Threonine phosphatase composed of a catalytic subunit (C), a structural subunit (A), and a variable regulatory subunit (B) (Figure 1 A; Janssens and Goris, 2001). The A and C subunits make up the enzymatic core of PP2A, while the variable B subunits competitively associate with the core unit, thus providing distinct substrate specificity for the enzyme (Janssens et al., 2008). Accordingly, the core subunits are ubiquitously expressed in all cell types, while B subunits expression is tissue and developmental stage-specific (Janssens et al., 2005; Janssens et al., 2008). My IP/MS experiment retuned the B subunit PP2A-B' as an aPKC-interacting proteins. The potential association of aPKC with the PP2A B-subunit suggests that aPKC maybe regulated by dephosphorylation and also raise the possibility that other B subunits may regulate aPKC in different cell types and developmental stages. Lysates used to perform these IP experiments came from embryos in various stages of embryonic development (0-12 hours) and contained several cell types.

Previous reports show that PP2A localizes with aPKC in vertebrate tight junction to regulate aPKC (Nunbhakdi-Craig et al., 2002). Also, PP2A can antagonize aPKC by dephosphorylating Par-1, a known aPKC substrate (Suzuki et al., 2004; Nam et al., 2007) thus indicating that PP2A can regulate aPKC directly and indirectly. In *Drosophila*, mutations in the B subunit, *Tws*, result in defects in mitotic progression (Mayer-Jaekel et al., 1993), fate misspecification in wing imaginal discs (Uemura et al., 1993) and in sensory organ precursor (SOP) progeny (Shiomi et al., 1994). Knockout of the catalytic

subunit (*microtubule star, mts*) is embryonic lethal (similar to vertebrate PP2A complete loss of function) however mosaic *mts* clones in *Drosophila* photoreceptors cause cell polarity defects (Wang et al, 2008). It is not clear whether any of the above defects result from aPKC misregulation. *mts* various defects are likely to be mediated by different, yet to be discovered, PP2A targets given the diversity of cellular processes that PP2A affects.

PP2A has been the focus of intense research, particularly for its role in cell growth and tumorigenesis. However, defining a clear role for PP2A in regulating cell growth and tumorigenesis has been difficult due to the lack of viable *PP2A* genetic mutants. It has been proposed that PP2A role in cell growth and tumorigenesis may reside within its B subunits: several mutations in B subunits or A subunits, which prevent binding of B subunits, have been associated with cancers (Chen et al., 2005; Li et al., 2007; Van Hoof and Goris, 2004). To investigate a role for PP2A in regulating aPKC, I focused on Twins (Tws), another B-subunit of PP2A for two reasons: several *tws* alleles were readily available, including a genetic mutant that would allow me to analyze Tws function clonally. Also, I had access to an antibody against Tws, which can prove to be extremely useful for biochemistry and immuno staining experiments. Here I show that Tws regulates cell polarity and cell fate in *Drosophila* brains.

Materials and Methods

Fly stocks and MARCM clones

The wild type fly stock was yellow white (y w). tws^{P} *FRT82B* was a gift from Ken Irvine (Rutgers). *Df(3)7732* flies were obtained from Bloomington stock center (Indiana).

Zygotic *tws* phenotypes were analyzed in *tws^P FRT82B /Df(3)7732* trans-heterozygotes. To generate positively marked MARCM wild type or *tws* clones, I mated *FRT82B* (Bloomington stock center, Indiana) or *tws^P FRT82B/TM6, Tb, Hu* to *y w hsFLP; tubP-GAL4 UAS-mCD8::GFP; FRT82B, tubP-GAL80/TM6,Tb, Hu* (Bello et al., 2008). Central brain clones were induced in 24-28 hours after larval hatching (ALH) larvae for 1 hour at 37°C, returned larvae to 25°C for 48 hours. For optic lobe clones, I induced clones in 48-52 hours ALH larvae for 1 hour at 37°C and aged them for 48 hours at 25°C.

Immuno-precipitations

For immunoprecipitation experiments, a 12 hours collection of y w embryos were homogenized in lysis buffer (50mM HEPES pH7.5, 150mMNaCl, .1% Tween-20, 1 mM EDTA, 2.5 mM EGTA, 10% Glycerol, supplemented with protease inhibitor tablets; Roche) to produce 1 mL of lysate. Lysates were pre-cleared with protein agarose-A beads for 1 hour at 4°C and subsequently divided equally (500 ⌈L each) in two eppendhorf tubes and incubated with 2 ⌈L of either anti-GFP or anti-Twins antibodies (a gift from Tadashi Uemura, Japan) for 4 hours at 4°C. Lysates were then incubated with protein agarose-A beads for 1 hour at room temperature. For pulldowns, beads were precipitated and washed 3 times in modified lysis buffer containing 1 mM NaCl, bound proteins were separated by SDS-PAGE, transferred onto nitrocellulose and probed for aPKC.

Antibodies and immuno-staining.

Neuroblasts polarity was examined with rabbit anti-aPKC, 1:1000 (Sigma); anti-Miranda, 1:500 (Doe lab); rabbit anti-phosphohistone H3, 1:1000 (Sigma, St.Louis, MO). For neuroblast self-renewal assays I used rabbit anti-Scrib, 1:2500 (Doe lab); rat monoclonal

anti-Dpn (Doe lab), 1:1; anti-Miranda, 1:500 (Doe lab); rabbit anti-GFP, 1:1000 (Sigma, St.Louis, MO). Secondary antibodies were obtained from Molecular Probes (Eugene, OR). Larval brains were dissected, fixed, and stained as described previously (Siller et al., 2005), and analyzed with a Bio-Rad Radiance 2000 or Leica TCS SP laser scanning confocal microscope using a 60x1.4 NA oil immersion objective. Images were processed with Illustrator software (Adobe).

Results

Twins and aPKC are parts of a same protein complex

The notion that PP2A may regulate aPKC in neuroblasts came from the observation that *Drosophila* aPKC contains several putative PP2A target sites that are conserved in worms and vertebrates (Figure 1 B) and PP2A can dephosphorylate aPKC in epithelial cells (Nunbhakdi-Craig et al., 2002), although it is not clear whether dephosphorylation of aPKC by PP2A is Tws-mediated. To confirm that Tws and aPKC are part of a same protein complex *in vivo,* as suggested by my IP/MS experiments, I performed pulldown experiments against a non-specific protein (GFP) or against aPKC. I found that aPKC and Tws co-immuprecipitate (Figure 1C), indicating that aPKC and Tws are part of a same protein complex and raising the possibility that Tws may target aPKC to PP2A for dephosphorylation.

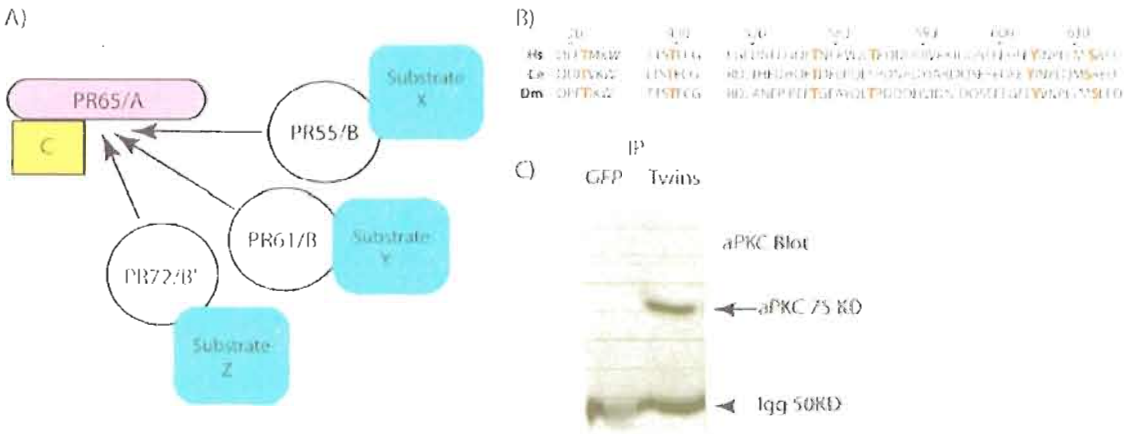


Figure 1. Tws and aPKC share the same protein complex

A) Schematic illustrating the complexity of PP2A: PP2A contain a catalytic (C) subunit, a structural subunit (A), and several variable regulatory subunits (B) that regulate the enzyme substrate specificity

B) *Drosophila* aPKC contains several putative PP2A target sites that are conserved in humans (Hs) and *C.elegans* (Ce)

C) Tws/aPKC co-immunoprecipitate (arrow) from embryonic lysates. Arrowhead denotes the antibody's Igg heavy chain.

Twins function is required to restrict aPKC to the neuroblast apical cortex

Next I examined the functional relevance of Tws/aPKC interaction in neuroblasts, more specifically I tested a role for Tws in regulating aPKC localization. To do so, aPKC localization was examined in wild type neuroblasts or *tws* mutant neuroblast clones induced in the dorsal-anterior-lateral (DAL) region of the central brain (Fig. 3G). Wild type clones showed aPKC crescents, aPKC was clearly absent from the basal cortex (Fig. 2A). In contrast, *tws* mutant neuroblast clones showed cortical patches of aPKC at the basal cortex in addition to an apical crescent (Fig 2D), suggesting that Tws normally inhibits aPKC localization to the basal cortex.

To address whether the ectopic pool of aPKC seen at the basal crescent is active (similar to *lgl, pins* mutants (Lee et al., 2006)or inactive (similar to *dap160* mutants, see chapter III), I analyze Miranda localization in Wild type neuroblast or *tws* mutant clones neuroblasts. Ectopic active aPKC displaces Miranda from the basal cortex, while inactive aPKC does not (Lee et al., 2006), thus Miranda localization can be diagnostic for determining the activity of ectopic aPKC. If Tws function is required for aPKC activity, I would expect Miranda to extend to the neuroblast apical cortex, similar to *apkc* mutant (Rolls et al., 2003). If however, loss of Tws function causes aPKC localization to the basal cortex without abolishing aPKC activity, I would expect *tws* mutant neuroblats to show cytoplasmic Miranda. Wild type neuroblasts showed strong basal Miranda (Fig. 2 B) but, Miranda was cytoplasmic in *tws* mutant neuroblast clones (Fig. 2 E), indicating

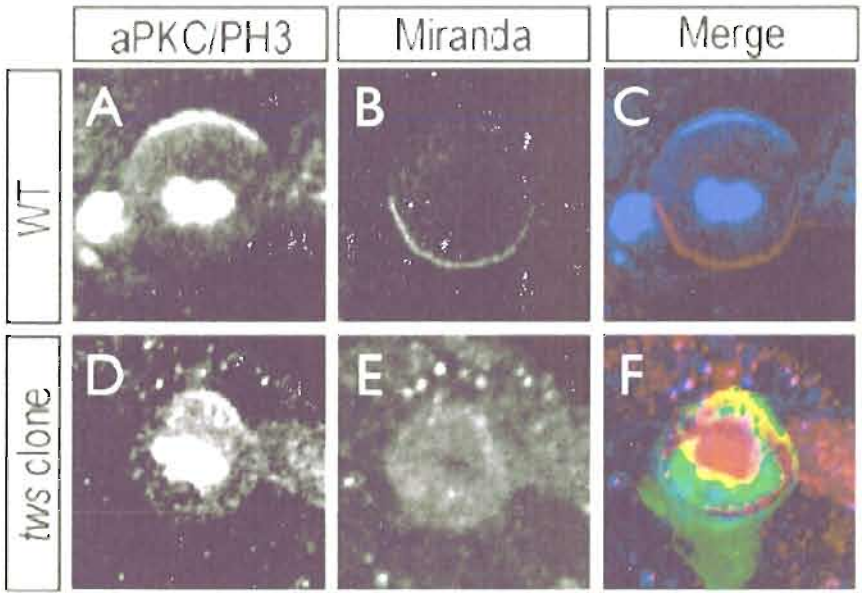


Figure 2. Tws is required to restrict active aPKC to the neuroblast apical cortex

(A-F) aPKC and Miranda localization in wild type or *tws* mutant neuroblast clones

A, C) aPKC is restricted apically in wild type neuroblasts, while (B, C) Miranda forms basal crescents

(D, F) *tws* mutant clones show ectopic aPKC and (E, F) cytoplasmic Miranda localization

that ectopic aPKC in *tws* mutant neuroblast clones is active. I conclude that Tws function is required to restrict/anchor active aPKC to the neuroblast apical cortex.

Tws suppresses neuroblast cell fate

The larval brain consists of two main populations of neuroblasts: central brain neuroblasts and optic lobe (OL) neuroblasts. Central brain neuroblasts, specified during embryogenesis, enter a quiescent period at the end of embryogenesis but progressively become re-activated or proliferative starting larval life and reach a maximum of ~100 neuroblasts per brain lobe (Lee et al., 2006; Urbach and technau, 2004). OL neuroblasts originate from the OL epithelium, where epithelial cells in the most medial region, but not the lateral region of the OL undergo a transition from epithelial fate to neuroblast fate (Egger et al., 2007). Similar to central brain neuroblasts, OL neuroblasts are polarized and divide to self-renew and give rise to a GMC, which will produce a pair of neurons (Egger et al., 2007).

Ectopic cortical aPKC activity in central brain neuroblasts is sufficient to cause the formation of supernumerary neuroblasts resulting from neuroblasts giving rise to two neuroblasts instead of one neuroblast and a GMC (Lee et al., 2006). It is not clear whether OL neuroblats are similarly susceptible to cortical aPKC.

First, to test whether ectopic aPKC observed in *tws* mutant neuroblasts is sufficient to induce formation of supernumerary neuroblasts in the central brain, I induced wild type or *tws* mutant single neuroblast clones and scored the number of in these regions divide to self-renew and produce transient amplifying GMCs that are Dpn^{+}

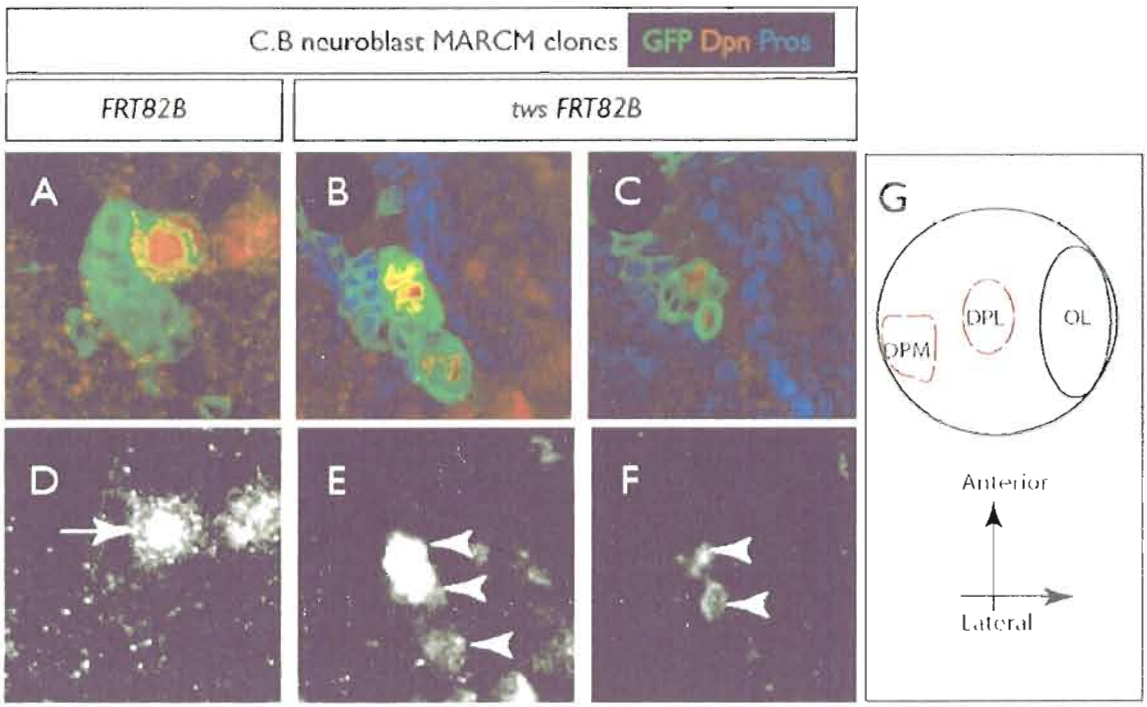


Figure 3. Tws suppresses neuroblast fate

A-F) wild type or *tws* single neuroblast clones stained with GFP (marking the clone), Deadpan (marking neuroblasts), and Prospero (marking GMC).

A,D) Single wild type neuroblast clones induced in non-DPM and non-DPL regions of the central brain showed a single neuroblast per clone (D, arrow).

(B,C,E,F) In contrast, similarly induced single *tws* mutant neuroblast clones contained up to four neuroblasts per clones (E and F, arrowheads).

G) A diagram depicting the DPM and DPL regions in the larval central brain.

(Boone and Doe, 2008; Bello et al., 2008). Wild type clones always contained a single Dpn^+ cell (Figure 3A, D) in contrast to *tws* clones, which contained up to four Dpn^+ cells per clone. Next I turned to larval brain OL to test whether Tws has a similar role there. OL epithelial cells undergo a fate transformation to adopt neuroblast fate, thus giving rise to OL neuroblats. This fate transition is spatially restricted to the most medial region of the OL. To test a role for Tws in epithelia to neuroblast fate transformation, I compared wild type brains to *tws* zygotic mutant brains for the presence of supernumerary neuroblasts in the OL epithelium using two independent neuroblast markers Dpn and Miranda. In wild type, neuroblasts were restricted to the medial edge of the OL (Figure 4 A, lines). The remainder of the OL tissue contained cells that were Dpn^-, $Miranda^-$, and had the characteristic columnar shape of epithelial cells (Figure 4 A, lines). In contrast, *tws* zygotic mutant OL epithelial cells were replaced by neuroblasts that populated the entire OL (Figure 4 B, lines), suggesting that Tws normally ensures the timely transition from epithelial fate to neuroblast fate in the OL.

Finally, to test whether Tws role in inhibiting neuroblast cell fate in the OL is cell-autonomous, similar to central brain neuroblasts, I induced wild type or *tws* single neuroblasts clones in the OL. I found that in contrast to wild type clones, which contained a single Dpn+ cell, *tws* mutant clones contained several Dpn^+ cells that expanded past the OL boundary (Figure 4 C vs. Figure 4 D, E). Preliminary data suggest that aPKC is ectopic in OL neuroblasts, similar to central brain neuroblasts (data not shown). Taken together I conclude that Tws inhibits neuroblast cell fate in OL epithelium is lineage-autonomous.

Figure 4. Tws regulates neuroblast fate in the larval optic lobe

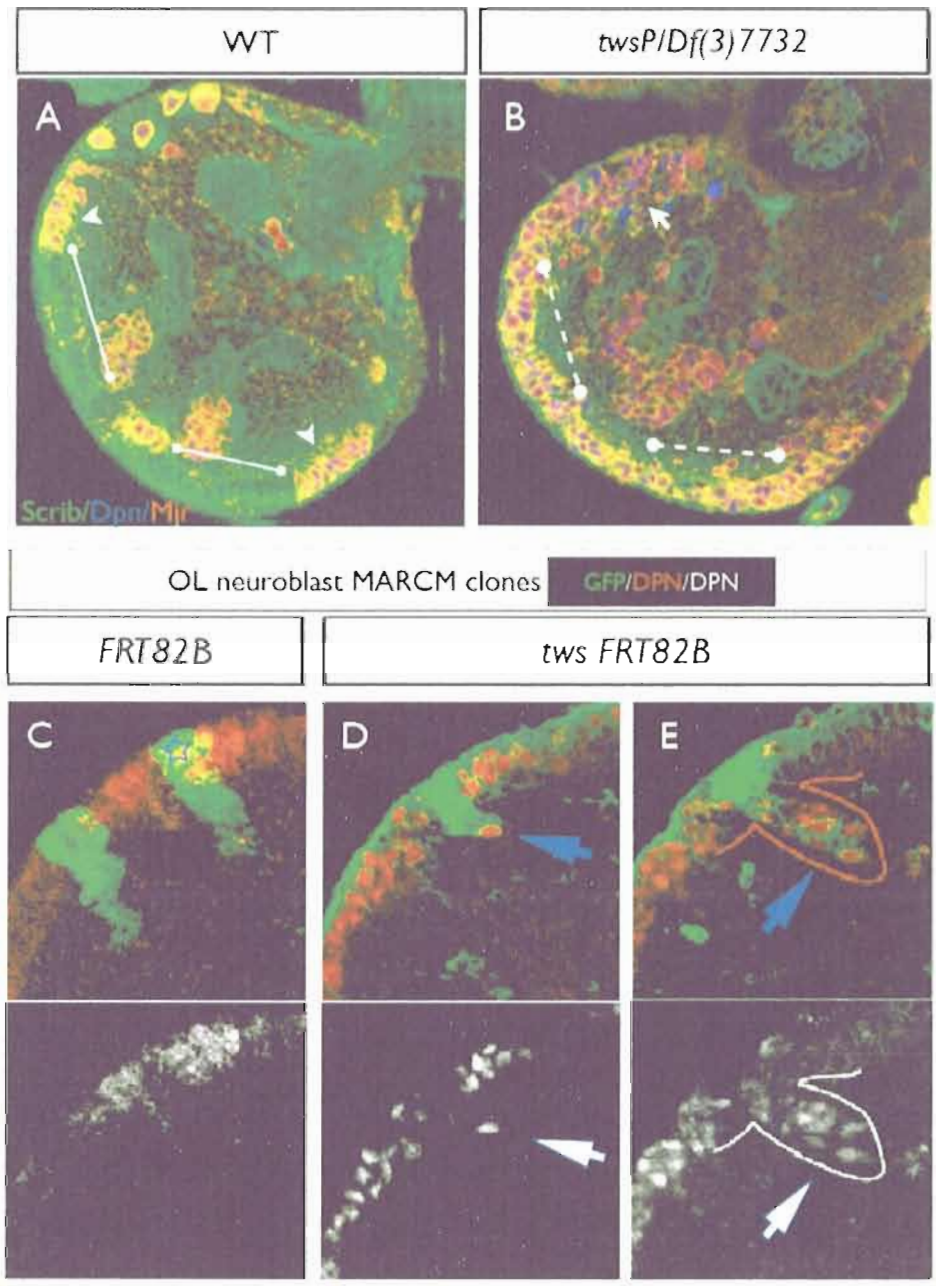
(A-E) Wild type or *tws* mutant optic lobe neuroblast clones: Deadpan and Miranda (Mir) mark the neuroblasts. Scribble (Scrib) labels the outline of the cells and GFP positively marks wild type or *tws* mutant clones.

A) In wild type, neuroblasts were restricted to the medial edge of the OL (A, arrowheads). As expected, the remainder of the OL tissue contained cells that were Dpn $^{-}$, Miranda $^{-}$, and displayed the characteristic columnar shape of epithelial cells (A, lines).

B) In contrast, *tws* zygotic mutant OL epithelial cells were replaced by neuroblasts that populated the entire OL, note that the majority of OL cells have lost their columnar shape (B, dashed-lines).

C) Wild type single OL neuroblast clones contain a single neuroblast (blue star)

D-E) *tws* single OL neuroblast clones contain neuroblasts outside of the OL cells layer (D and E, arrowheads)


WT
twsP/Df(3)7732
A
B
Scrib/Dpn/Mir
OL neuroblast MARCM clones
GFP/DPN/DPN
FRT82B
tws FRT82B
C
D
E

Conclusions

Cells use reversible phosphorylation as a means to regulate diverse signal transduction pathways. In *Drosophila,* several kinases, including aPKC regulate neuroblast cell polarity and self-renewal (Chia et al., 2008) however the identity of the counter acting phosphatase, which opposes these kinases and/or their substrates is unclear. In this study I show that Tws, a B subunit of PP2A promotes GMC differentiation/inhibit neuroblast self-renewal by regulating aPKC localization. Tws is also required for timely epithelial to neuroblast fate transition in OL.

How does Tws regulate aPKC localization? Tws may regulate aPKC localization by targeting aPKC to PP2A core structure for dephosphorylation. In this model, dephosphorylation of aPKC at specific sites would affect aPKC subcellular distribution. More specifically, it would restrict aPKC localization to the neuroblast apical cortex. An alternative hypothesis is that Tws regulates aPKC localization independent of PP2A enzymatic activity. Tws may regulate aPKC localization by stabilizing aPKC interaction with other apical proteins thus restricting it there or by preventing aPKC binding to basal protein(s). To distinguish between a dephosphorylation-dependent and a dephosphorylation-independent mode of aPKC regulation, it will be desirable to compare aPKC phosphorylation state in wild type with *tws* mutant brains. The prediction here is that if Tws normally facilitate aPKC dephosophorylation by PP2A, then one might observe a hyper phosphorylated aPKC in Tws mutant brain lysates compared to wild type lysate. More importantly, it would be informative to compare aPKC localization in mutant clones of PP2A catalytic

subunit (*mts*) to mutant clones of *tws*. If *mts* clones phenocopy *tws* clones in terms of aPKC mislocalization defects, it will strongly suggest that Tws regulation of aPKC localization is dependent on PP2A enzymatic activity. A negative outcome to the two experiments described above would suggest that Tws regulation is PP2A-independent, in which case it would be interesting to analyze Tws and aPKC co-localization in neuroblasts. If Tws restricts aPKC to the apical cortex by stabilizing it there, then one might expect to see Tws protein co-localize with aPKC in a apical crescent. If however, Tws achieves aPKC restriction to the apical cortex by suppressing aPKC binding to basal proteins, I expect Tws to show basal cortex localization. It should however not be surprising if Tws is cytoplasmic in neuroblasts, as Tws may exclude aPKC from the basal cortex by sequestering aPKC in the basal cytoplasm. A third possibility is that Tws regulates aPKC localization to the apical cortex utilizing both mechanisms: apical stabilization and basal cortex exclusion. Here I would expect Tws to be cortical, perhaps with an apical enrichment. Elucidating the details underlying Tws regulation of aPKC will greatly improve our understanding of aPKC regulation.

In summary, aPKC has been implicated in various cellular processes, including apoptosis, cell growth, and differentiation in vertebrates as well as invertebrates. Despite the growing interest on aPKC, relatively little is known about how aPKC is regulated. Using *Drosophila* powerful genetics, I have established three distinct modes of aPKC regulation in the context of neuroblasts cell polarity and self-renewal: *i)* Cdc42 activates aPKC via the scaffolding protein Par-6, *ii)* In contrast to Cdc42

indirect stimulation of aPKC, Dap160 directly binds and activates aPKC, *iii)* The regulatory subunit of the phosphatase PP2A, Twins, regulates aPKC localization. These findings enhance our collective understanding of aPKC regulation and are likely to provide the medical research community new insights into how aPKC-mediated human pathologies arise. Of particular note, it will be interesting to investigate aPKC/Tws or aPKC/Dap160 relationships in various tumor models where aPKC, Dap160, and PP2A have been individually implicated.

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