ATYPICAL PROTEIN KINASE C REGULATES *DROSOPHILA* NEUROBLAST POLARITY AND CELL-FATE SPECIFICATION

by

SCOTT X. ATWOOD

A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

September 2008

University of Oregon Graduate School

Confirmation of Approval and Acceptance of Dissertation prepared by:

Scott Atwood

Title:

"Atypical Protein Kinase C regulates Drosophila neuroblast polarity and cell-fate specification"

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Karen Guillemin, Chairperson, Biology
Kenneth Prehoda, Advisor, Chemistry
Alice Barkan, Member, Biology
Christopher Doe, Member, Biology
Tom Stevens, Outside Member, Chemistry

and Richard Linton, Vice President for Research and Graduate Studies/Dean of the Graduate School for the University of Oregon.

September 6, 2008

Original approval signatures are on file with the Graduate School and the University of Oregon Libraries.

An Abstract of the Dissertation of

Scott X. Atwood for the degree of Doctor of Philosophy

in the Department of Biology to be taken September 2008

Title: ATYPICAL PROTEIN KINASE C REGULATES *DROSOPHILA* NEUROBLAST POLARITY AND CELL-FATE SPECIFICATION

Approved: _____
Dr. Kenneth Prehoda

Cellular polarity is a biological mechanism that is conserved across metazoa and is used in many different biological processes, one of which is stem cell self-renewal and differentiation. Stem cells generate cellular diversity during development by polarizing molecular determinants responsible for directing one daughter cell to maintain stem cell-like qualities and the other daughter cell to initiate a specific cell fate. The stem cell self-renewal versus differentiation choice is critical to avoid overproliferation of stem cells and tumor formation or underdevelopment of tissues and early animal death. *Drosophila* neural stem cells (neuroblasts) undergo asymmetric cell division (ACD) to populate the fly central nervous system and provide an excellent model system to study processes involving cellular polarity, ACD, stem cell self-renewal, and differentiation. Neuroblasts divide unequally to produce a large, apical self-renewing neuroblast and a small, basal ganglion

mother cell that goes on to divide and form two neurons or glia. In this way, a small population of neuroblasts can give rise to thousands of neurons and glia to generate a functional central nervous system.

Atypical Protein Kinase C (aPKC) is critical to establish and maintain neuroblast polarity, ACD, stem cell self-renewal, and differentiation. aPKC is part of the evolutionarily conserved Par complex, whose other members include Bazooka and Par-6, and they localize to the neuroblast apical cortex and function to restrict cell-fate determinants into one daughter cell. How aPKC is asymmetrically localized and how its activity translates into cell-fate specification are of incredible importance as *apkc* mutants where localization is disrupted no longer segregate cell-fate determinants. This work will show that Cdc42 recruits the Par-6/aPKC complex to the neuroblast apical cortex independent of Bazooka. Once there, aPKC phosphorylates the cell-fate determinant Miranda to exclude it from the apical cortex and restrict it basally. Par-6 and Cdc42 regulate aPKC kinase activity though inter- and intramolecular interactions that allow high aPKC kinase activity at the apical cortex and suppressed activity elsewhere. Cdc42 also functions to keep aPKC asymmetrically localized by recruiting the PAK kinase Mushroom bodies tiny to regulate cortical actin and provide binding sites for cortical polarity determinants.

This dissertation includes previously published co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Scott X. Atwood

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, Oregon

University of Utah, Salt Lake City, Utah

DEGREES AWARDED:

Doctor of Philosophy in Molecular Biology, University of Oregon, 2008

Bachelor of Science in Biology, University of Utah, 2003

AREAS OF SPECIAL INTEREST:

Cell Polarity

Stem Cell Biology

Kinase Regulation

GRANTS, AWARDS AND HONORS:

Developmental Biology Training Grant, NIH, 2005-2008

Faculty of 1000 Recommendation, Cdc42 acts downstream of Bazooka to regulate neuroblast polarity through Par-6-aPKC, 2007

PUBLICATIONS:

Atwood, S.X. and Prehoda, K.E. (2008). Phosphorylation-mediated cortical displacement of fate determinants by aPKC during neuroblast asymmetric cell division. *Nature Cell Biology*, submitted.

Atwood, S. X., Chabu, C., Penkert, R. R., Doe, C. Q. and Prehoda, K. E. (2007). Cdc42 acts downstream of Bazooka to regulate neuroblast polarity through Par-6 aPKC. *J Cell Sci* **120**, 3200-6.

ACKNOWLEDGMENTS

I wish to express sincere gratitude to my advisor, Dr. Ken Prehoda, for his patient guidance in shaping the way I think and approach scientific endeavors. I thank my committee members for their support and critiques throughout my graduate career. My fellow lab members have been instrumental in the completion of my work and I thank them for providing a wonderful lab atmosphere. Special thanks go to Dr. Rick Nipper, Dr. Rhonda Newman, and Dr. Jason Boone for their insightful ideas that helped me gain a better understanding of neuroblast polarity. I whole-heartedly thank Dr. Chris Doe for many helpful collaborations and expertise in preparing various manuscripts. His lab members have been incredibly helpful over the years, and Dr. Yves Chabu and Ryan Anderson deserve special recognition for collaborations on the Cdc42 and Lgl projects. I received funding for this work from a National Institute of Health Developmental Biology Training Grant (5-T32-HD07348), and a Damon Runyon Scholar award and National Institute of Health grant (GM068032) awarded to Ken Prehoda.

To Mom and Dad for your love and support, and to Jenn for your patience and understanding

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Cellular polarity is a biological mechanism that is conserved across metazoa and is used in many different biological processes such as epithelial barrier functions, cell-to-cell contacts, cellular motility, as well as stem cell self-renewal and differentiation. Stem cells generate cellular diversity during development by polarizing molecular determinants responsible for directing one daughter cell to maintain progenitor-like qualities – termed self-renewal – and the other daughter cell to suppress the stem cell program and initiate a specific cell-fate – termed differentiation. The stem cell self-renewal versus differentiation choice is critical to avoid overproliferation of stem cells and tumor formation or underdevelopment of tissues and early death. Studies in the stem cell and cellular polarity fields shed light on important processes such as embryogenesis, organ development, cancer biology, developmental and congenital defects and diseases, and stem cell therapeutics.

Drosophila neural stem cells (neuroblasts) undergo asymmetric cell division (ACD) to populate the fly central nervous system and provide an excellent model system to study processes involving cellular polarity, ACD, and stem cell self-renewal and differentiation. Neuroblasts divide unequally to produce a large, apical self-renewing

neuroblast and a small, basal ganglion mother cell (GMC) that goes on to divide and form two neurons or glia. In this way, a small population of neuroblasts can give rise to thousands of neurons and glia to generate a functional central nervous system. Neuroblasts receive many intrinsic and extrinsic cues to know when and where to divide. Cellular polarity and spindle orientation are critical intrinsic cues that establish ACD and cell-fate segregation whereas the cellular niche is important for maintaining neuroblast self-renewal and proliferation. Many molecular components are necessary for these processes and are discussed below. However, one protein in particular, Atypical Protein Kinase C (aPKC), seems to drive neuroblast polarity, ACD, and the decision between self-renewal and differentiation. The proper localization and activity of aPKC is critical for all these processes and this work will delve into the mechanisms that regulate aPKC activity and localization.

Neurogenesis and cell-fate specification

Drosophila neuroblasts are born from neuroepithelial cells in the embryo during neurogenesis (Doe, 2008; Egger et al., 2008). Neuroepithelial cells line the periphery of the early embryo and can form either neuroblasts or epithelia. The decision to form either two cell types depends on the transmembrane receptor Notch activity, which promotes epithelial cell fate and inhibits neuroblast formation (Artavanis-Tsakonas and Simpson, 1991). Loss of Notch function causes severe defects that result in the formation of extra neuroblasts at the expense of epithelia. Several proneural transcription factor genes, *achaete*, *scute*, and *lethal of scute*, repress Notch activity and promote neuroblast cell fate

by positively regulating Delta expression. Delta, a transmembrane ligand, binds and activates Notch in neighboring cells to laterally inhibit neuroblast fate, thus ensuring that cells with high levels of Delta expression and low levels of Notch activity become neuroblasts while limiting neuroblast populations. Other factors are also involved in the decision to promote neuroblast fate in a subset of neuroepithelia, such as SoxN and Dichaete, two members of the SoxB group of transcription factors (Buescher et al., 2002; Overton et al., 2002).

After delamination from the neuroectoderm, neuroblasts begin to divide asymmetrically to generate a larger, self-renewing neuroblast and a smaller GMC. A handful of embryonic and larval neuroblasts can generate thousands of neurons and glia that give rise to the fly central nervous system (Ito and Hotta, 1992). While some neural progenitors can divide symmetrically to expand the progenitor population, such as those found in the larval brain optic lobe, most divide asymmetrically (Egger et al., 2007). Although several factors have been shown to promote neuroblast self-renewal and will be discussed below, no known transcription factors have been implicated in this process even though several have been identified in mammalian neural stem cell systems suggesting that more work needs to be done to fully understand the process of self-renewal in *Drosophila* (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). More is known about neuronal differentiation as this process is promoted by the transcription factor Prospero (Pros), translational repressor Brain tumor (Brat), and Numb (reviewed in Knoblich, 2008). Pros represses genes involved in neuroblast self-renewal and cell-cycle, and activates genes involved in terminal differentiation (Choksi et al., 2006). The

function of Brat is less known but seems to be required to restrain cell growth, partly by blocking *myc* translation (Betschinger et al., 2006). *pros* and *brat* mutants cause neuroblast overproliferation defects and tumors and can exacerbate each others' phenotype indicating they act in distinct, yet redundant pathways (Betschinger et al., 2006; Lee et al., 2006c). Numb functions as a repressor of Notch to promote self-renewal in the neuroblast and inhibit self-renewal in the GMC (Yoon and Gaiano, 2005). *numb* mutants also cause neuroblast overproliferation defects and tumors that are similar to *pros* and *brat* mutants (Lee et al., 2006c; Wang et al., 2006). A critical aspect of neuroblast ACD is to asymmetrically segregate Pros, Brat, and Numb into the GMC as failure to do so results in premature differentiation of the neuroblast and organismal death.

Establishing cortical polarity and spindle orientation

Embryonic neuroblast polarity and its orientation of divisions is originally established from proteins expressed in epithelia and are inherited when the neuroblast delaminates from the neuroepithelial layer. Bazooka (Baz; Par-3) is the first known apical polarity component recruited to the apical cortex of both neuroblasts and epithelia (Kuchinke, 1998). Baz is a scaffolding protein that recruits the other so-called "Par complex" members Par-6 and aPKC to the neuroblast apical cortex through asymmetric activation of the Rho GTPase Cdc42 (Atwood et al., 2007). Cdc42 recruits the Par-6/aPKC complex by interacting with the Par-6 CRIB domain (Atwood et al., 2007). Par-6 also contains a PB1 domain that interacts with the PB1 domain of aPKC (Noda et al.,

2003), and a PDZ domain that interacts with the first of three PDZ domains within Par-3 (Joberty et al., 2000). aPKC is a kinase that phosphorylates many proteins involved in cellular polarity, one of which is Baz (Nagai-Tamai et al., 2002). The Par complex members were originally identified in *C. elegans* as regulators of early oocyte polarity, but have since been shown to be conserved across metazoa and involved in essentially all biological processes that involve cell polarity.

One of the functions of the Par complex in neuroblasts is to direct the segregation of the cell-fate determinants Pros, Brat, and Numb. Pros and Brat bind the coiled-coiled protein Miranda (Mira)(Betschinger et al., 2006; Ikeshima-Kataoka et al., 1997; Lee et al., 2006c; Shen et al., 1997) and Mira transports these cargoes to the basal cortex in response to the apical localization of Par-6/aPKC (Betschinger et al., 2003). Numb binds the coiled-coil protein Partner of Numb (Pon)(Lu et al., 1998), however, redundant mechanisms seem to restrict Numb to the basal cortex as removal of Pon only causes a delay in Numb basal localization, which eventually localizes during anaphase and telophase (Wang et al., 2007a). *baz*, *cdc42*, *par6*, and *aPKC* mutant neuroblasts are unable to asymmetrically segregate Mira and Numb, who are uniformly cortical, and result in the premature death of the animal during development (Atwood et al., 2007; Petronczki and Knoblich, 2001; Rolls et al., 2003; Schober et al., 1999). The main mechanism by which Numb is asymmetrically localized is through phosphorylation by aPKC, which dissociates Numb from the apical cell cortex leading to its segregation to the basal cortex (Smith et al., 2007). Although, it seems Pon plays a minor role early in the basal localization of Numb early in mitosis as phosphorylation of Pon by the cell-

cycle kinase Polo can restrict Pon/Numb to the basal cortex (Wang et al., 2007a). How Mira, and its cargo Pros and Brat, segregates to the basal cortex remains unresolved.

In addition to the Par-complex, the tumor suppressors Lethal giant larvae (Lgl) and Discs Large (Dlg) are required for basal targeting of Mira and Numb. Dlg localizes to the cell cortex and is apically enriched during mitosis (Peng et al., 2000; Siegrist and Doe, 2005), whereas the localization of Lgl is more controversial as it has been reported in several locations (Albertson and Doe, 2003; Betschinger et al., 2003; Peng et al., 2000) but is likely restricted to the basal cortex (communicated by Jason Boone and Rhonda Newman). The localization of Lgl is regulated by aPKC, which phosphorylates Lgl to displace it from the apical cortex of neuroblasts (Betschinger et al., 2003). aPKC also phosphorylates Dlg, but how this affects localization or function remains to be determined (unpublished results). *lgl* mutant neuroblasts are defective in Mira and Numb localization, which are no longer associated with the cell cortex but are cytoplasmic and results in overproliferation of neuroblasts and tumor formation (Ohshiro et al., 2000). However, these defects are likely due to defects in apical polarity as aPKC and Par-6, but not Baz, are also ectopically localized around the cell cortex suggesting Par-6/aPKC activity displaces Mira and Numb (Smith et al., 2007). *dlg* mutant neuroblasts also show cortical Mira and Numb, along with neuroblast overproliferation defects and tumor formation (Peng et al., 2000). However, the molecular mechanism behind these defects remains unclear but as aPKC and Dlg interact (unpublished results), one likely explanation could be repression of aPKC kinase activity by mutant Dlg protein.

Baz also links neuroblast polarity to mitotic spindle orientation through the binding and recruitment of the neuroblast-specific protein Inscuteable (Insc) to the apical cortex (Schober et al., 1999; Wodarz et al., 1999). Insc recruits Partner of Inscuteable (Pins) to the apical cortex (Yu et al., 2000) where Pins binds the heterotrimeric G-protein member Gai and microtubule binding protein Mushroom body defect (Mud) to orient the mitotic spindle (Izumi et al., 2006; Schaefer et al., 2000; Siller et al., 2006). Pins undergoes an intramolecular interaction upon binding of Gai, which increases the affinity for Mud binding, and anchors astral microtubules to the apical cortex through direct binding to Mud (Nipper et al., 2007). *insc* and *pins* mutants result in misaligned mitotic spindles and neuroblast underproliferation defects that result in premature death of the animal, presumably because apical polarity is disrupted (Kraut et al., 1996; Schaefer et al., 2000; Yu et al., 2000). *mud* mutants also result in misaligned mitotic spindles but have neuroblast overproliferation defects as apical polarity is maintained increasing the number of cells inheriting cortical Par complex members responsible for self-renewal (Izumi et al., 2006; Siller et al., 2006). *gai* mutants have misaligned mitotic spindles as well, but have other complex defects that have not been explained (Lee et al., 2006b).

Dlg is also involved in mitotic spindle orientation. Dlg recruits microtubles by binding Kinesin heavy chain 73 (Khc-73), which is localized at the plus ends of astral microtubules. The Dlg/Khc-73 complex also binds Pins to induce Pins/Gai cortical polarity in alignment with the mitotic spindle. *dlg* mutants have slight mitotic spindle alignment defects (Siegrist and Doe, 2005) as well as defects in cell-fate segregation at metaphase (Peng et al., 2000), whereas knocking-down *khc-73* results in more robust

spindle defects and exaggerates *insc* mutant polarity defects (Siegrist and Doe, 2005). Dlg binds the linker region within Pins (Bellaiche et al., 2001) and it seems that both the Pins/Gai/Mud and Dlg/Khc-73 pathways are necessary for wild-type mitotic spindle alignment (communicated by Chris Johnston).

Neuroblast niche

Extrinsic cues from the surrounding tissues, as well as intrinsic cues from inside the cell, play roles in determining the axis of division and position of apical polarity in neuroblasts. Embryonic neuroblasts are still in contact with their overlying epithelia early in development and seem to receive positional cues to orient both polarity and mitotic spindle alignment. Neuroblasts grown in cell culture, but still in contact with epithelial cells, are able to maintain orientation of the mitotic spindle through several rounds of division (Siegrist and Doe, 2006). Neuroblasts not in contact with other cells are unable to maintain the orientation of their mitotic spindles and produce GMCs in seemingly random directions. Analogously, embryonic neuroblasts that later lose contact with the overlying epithelium partially lose the ability to orient their mitotic spindles (Egger et al., 2008). How neuroblasts orient their polarity and mitotic spindle later in development when contact with the epithelium is lost is unknown.

Glial cells contact neuroblasts later in embryonic development and this might provide the positional cue necessary to orient polarity and the axis of division (Doe, 2008). Glial cells secrete the glycoprotein Anachronism (Ana) to keep neuroblasts quiescent during late embryogenesis to early larval stages. Loss of contact between glial

cells and their neuroblast counterparts by expression of dominant-negative E-cadherin results in fewer proliferating neuroblasts (Dumstrei et al., 2003). The glycoprotein Activin is also expressed by glia and neuroblasts that lack Activin receptors have defects in neuroblast proliferation but not self-renewal (Zhu et al., 2008). Neuroblasts with decreased expression of Fibroblast growth factor (FGF) receptor, Hedgehog (Hh), or Perlecan – which binds either Hh or FGF – also results in a decrease in proliferating neuroblasts (Park et al., 2003). Notch signaling, as discussed earlier, seems to regulate neuroblast self-renewal. Reducing Notch activity decreases central brain neuroblast numbers (Wang et al., 2007a), whereas increasing Notch activity increases brain neuroblast numbers (Lee et al., 2006a; Wang et al., 2006).

Questions remaining in neuroblast ACD

Crucial questions involving ACD of Drosophila neuroblasts are addressed in the following chapters.

How do neuroblasts establish apical and basal-lateral domains?

How does initial apical polarity from Baz recruit other apical polarity members?

How does Par complex activity translate to cell-fate determinant segregation?

How are Par complex proteins regulated at the molecular level?

Does the internal structure of the neuroblast facilitate cortical polarity?

Surprisingly, all of these questions involve either the recruitment, or activity, of aPKC. aPKC activity not only has to be finely tuned, but its localization is crucial for its function. *aPKC* zygotic mutant animals die in early larval stages. *aPKC* mutant

neuroblasts have defects in polarity such as cortical Mira and cytoplasmic Par-6, indicating aPKC is necessary for segregation of cell-fate determinants and apical localization of Par-6 (Rolls et al., 2003). Cortical aPKC activity induced by expression of aPKCcaax (aPKC with a C-terminal isoprenylation motif to allow insertion into membranes) results in cytoplasmic Mira, massive neuroblast overproliferation defects, and tumors (Lee et al., 2006b). Expression of a kinase-dead version of aPKCcaax does not cause overproliferation or polarity defects indicating aPKC cortical activity promotes neuroblast self-renewal. These experiments suggest an incredibly important role for properly localizing and regulating aPKC activity. In the next four chapters, aPKC's role in neuroblast polarity and cell-fate specification will be discussed. Chapter II will show how aPKC is recruited to the cortex of neuroblasts and give a small taste of how aPKC kinase activity is regulated by Par-6 and Cdc42. This work has been previously published with other authors. Chapter III will show how aPKC activity is restricted to the apical cortex and how aPKC activity translates into cell-fate determinant segregation. Chapter IV will delve into the molecular mechanisms of aPKC kinase activity regulation by Par-6 and Cdc42. Chapter V will address how Cdc42 regulates cortical actin and show actin's role in maintaining cortical polarity. Finally, chapter VI will provide the relevance of this work to the field of cell polarity, asymmetric cell division, and stem cell self-renewal and differentiation.

CHAPTER II

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

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 (Egger et al., 2008; 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 (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

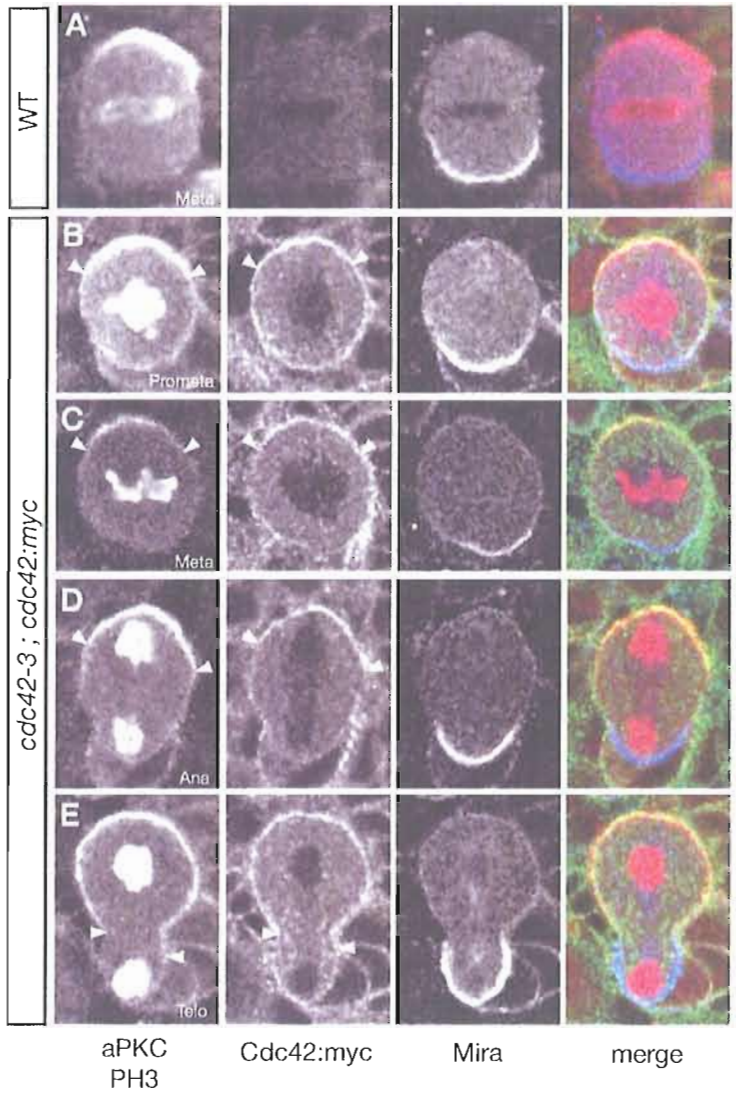


Figure 1. Cdc42 is enriched at the apical neuroblast cortex. (A) Wild-type central brain neuroblasts at 120 h after larval hatching (ALH). Normal apical and basal protein localization is shown with background c-myc staining. (B-E) *cdc42-3* central brain neuroblasts at 96 h ALH expressing Cdc42:myc under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

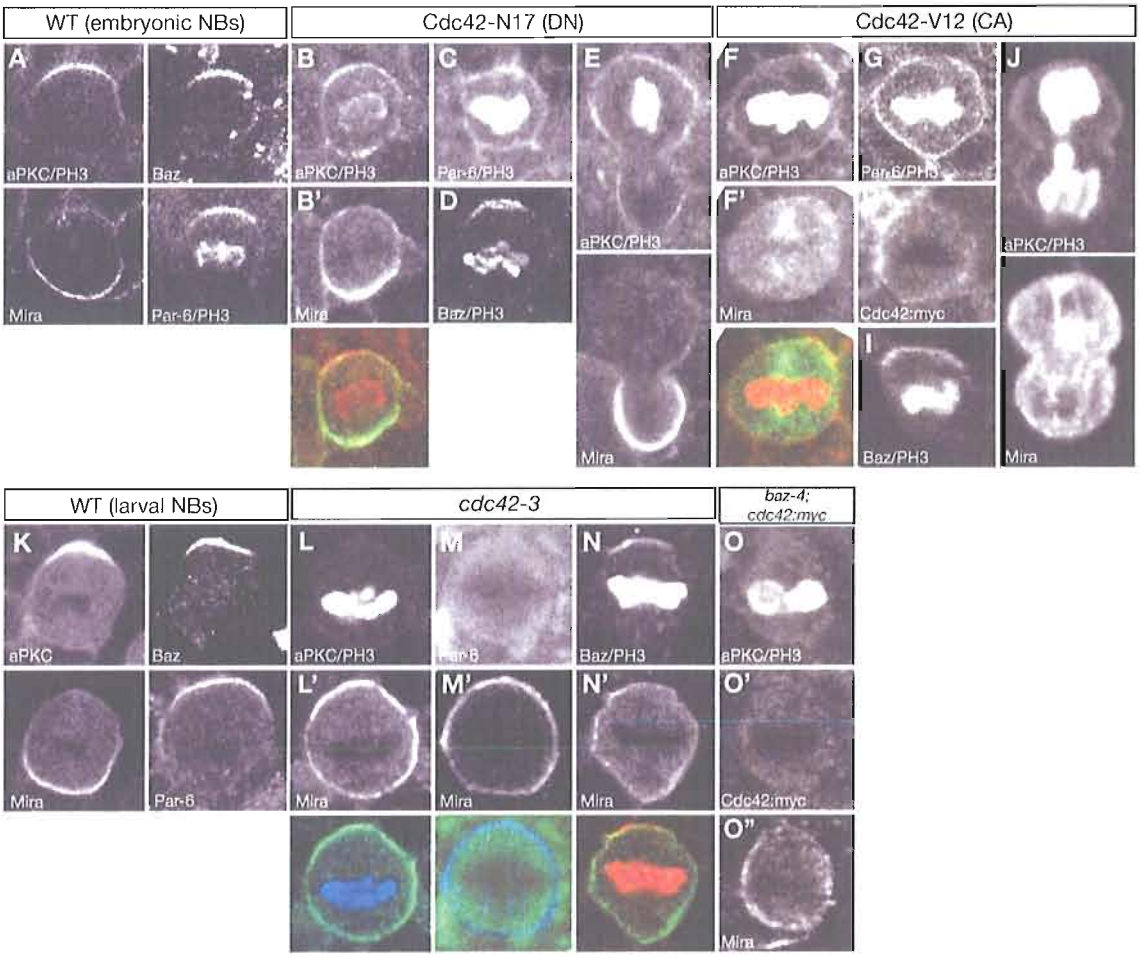
reasoned that using a gal4 line with earlier expression might increase the penetrance of the phenotype. Indeed, when we use *worniu-gal4* — which exhibits neuroblast-specific, high-level expression from the time of neuroblast formation (Albertson and Doe, 2003) — we find a dramatic increase in the percentage 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 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-

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
Par-6/PH3
B'
Mira
D
Baz/PH3
C
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
baz-4; cdc42:myc
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

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.

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.

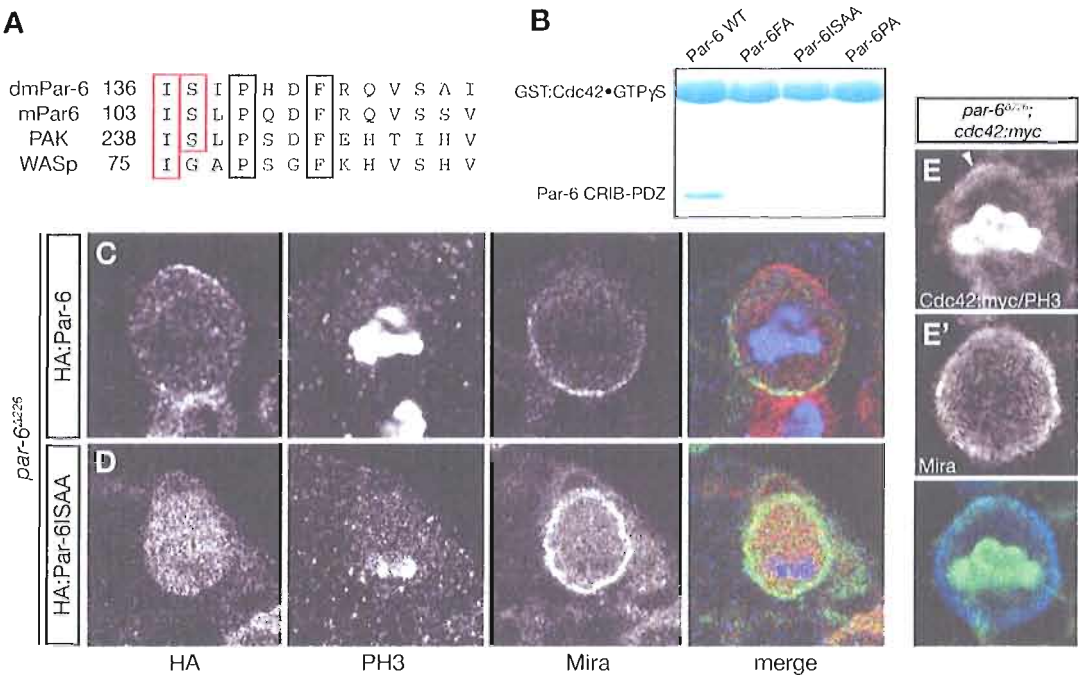


Figure 3. Cdc42/Par-6 interaction is necessary for neuroblast polarity. (A) Alignment of the Par-6 semi-CRIB domain with CRIB domains from other proteins. Mutated residues are boxed and the residues mutated in the Par-6^{ISAA} transgene are boxed in red. (B) The ISAA mutation disrupts Cdc42 binding to the Par-6 CRIB-PDZ domain. The extent of binding between a glutathione-S-transferase (GST) fusion of GTPgS 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).

We next tested the function of Par-6^{ISAA} in neuroblast polarity. We find that wild-type HA:Par-6 can effectively rescue *par-6* mutants for apical aPKC localization and basal Mira localization (Fig. 3C; data not shown), but that HA:Par-6^{ISAA} shows cytoplasmic aPKC and uniform cortical Mira (Fig. 3D; data not shown). This is identical

to the *cdc42-3* mutant phenotype (Fig. 2). We conclude that Cdc42 binds the Par-6 CRIB-PDZ domain, that this interaction is necessary and sufficient to recruit Par-6 to the neuroblast cortex, and that Cdc42 acts via Par-6 to regulate neuroblast polarity and asymmetric cell division.

Although Baz can localize to the apical cortex 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

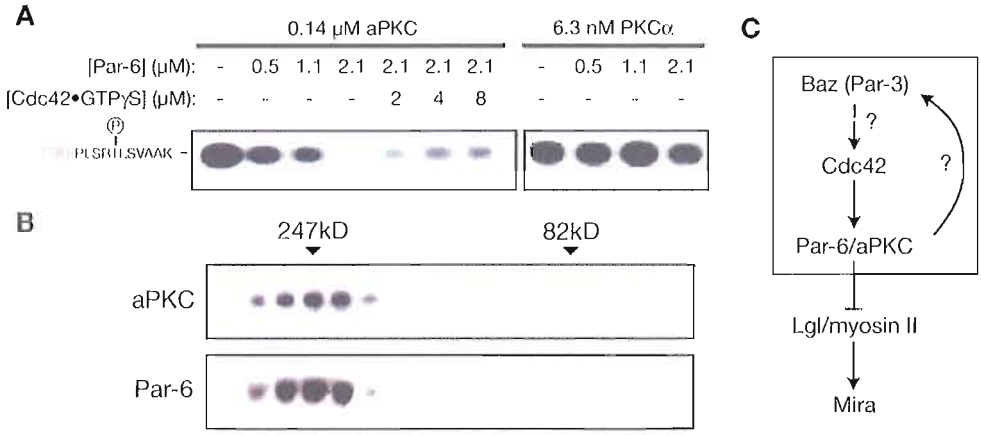


Figure 4. Par-6 represses while Cdc42 partially relieves aPKC kinase activity. (A) Kinase activity of aPKC, Par-6/aPKC, and Cdc42/Par-6/aPKC complexes. The high intrinsic kinase activity of aPKC, expressed and purified from HEK 293 cells, is efficiently repressed by addition of full-length Par-6. Par-6 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.

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 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.

Bridge to Chapter III

Chapter II discussed how aPKC is recruited to the neuroblast cortex. Cdc42 localizes Par-6/aPKC to the cortex downstream of Baz. Cdc42 has an additional function in activating aPKC kinase activity from the repressed Par-6/aPKC complex. Chapter III will go on to discuss how aPKC is restricted to the apical neuroblast cortex and how aPKC activity translates into cell-fate determinant segregation.

CHAPTER III

PHOSPHORYLATION-MEDIATED CORTICAL DISPLACEMENT OF FATE DETERMINANTS BY APKC DURING NEUROBLAST ASYMMETRIC CELL DIVISION

Introduction

Asymmetric cell divisions generate daughter cells with distinct fates by polarizing fate determinants into separate cortical domains. Atypical protein kinase C (aPKC) is an evolutionarily conserved regulator of cell polarity. In *Drosophila* neuroblasts, apically restricted aPKC is required for segregation of neuronal differentiation factors such as Miranda and its cargoes Prospero and Brat to the basal cortical domain. It has been proposed that aPKC regulates Miranda asymmetry by a cascade of repressive interactions (aPKC –| Lgl –| Myosin II –| Miranda). Here we provide biochemical, cellular, and genetic data to show that aPKC directly phosphorylates Miranda to exclude it from the apical cortex. Furthermore, we show that the tumor suppressor Lethal giant larvae (Lgl) displaces aPKC and its binding partner Par-6 from the basal cortex independently of Bazooka, thus explaining its role in promoting Miranda asymmetry. This simple model is sufficient to explain aPKC and Lgl mediated neuroblast cell polarity and sibling cell fate differences.

Drosophila neuroblasts divide to form a neuroblast and a ganglion mother cell (which typically divides once to generate two postmitotic neurons), and are an excellent model system for studying self-renewal and differentiation (Doe, 2008; Knoblich, 2008). Recent work has made it clear that the asymmetric activity of aPKC is a central facet of polarity in neuroblasts and many other polarized systems such as epithelia. Recruitment of aPKC to the apical neuroblast cortex occurs through the combined action of Bazooka (Baz; aka Par-3), Par-6, and Cdc42. Apically localized Baz recruits GTP-bound Cdc42, which in turn binds the semi-CRIB domain of Par-6 to recruit aPKC (Atwood et al., 2007; Kay and Hunter, 2001; Lin et al., 2000; Noda et al., 2003; Schober et al., 1999). However, two fundamental aspects of aPKC mediated polarity, namely how aPKC activity is restricted to the apical neuroblast cortex and how this activity is translated into the polarization of downstream components, have been unclear.

A complex model has been proposed to explain how aPKC activity leads to mutually exclusive cortical domains (Barros et al., 2003). Several molecules have been identified that are thought to function downstream of aPKC to polarize fate determinants. Apical aPKC phosphorylates the tumor suppressor Lgl (Betschinger et al., 2003), which inhibits its ability to repress Myosin II (Barros et al., 2003). Myosin II has been proposed to physically displace Miranda from the cortex, "pushing" it from the apical to basal cortex (Barros et al., 2003). This leads to a complex pathway in which aPKC phosphorylates Lgl, preventing its inhibition of Myosin II, ultimately removing Miranda from the cortex at sites of aPKC activity. However, it is unknown how Lgl or Myosin II might be involved in Miranda cortical localization. Furthermore, several key observations

are inconsistent with this model, including the normal polarity observed in *zip* mutants (Peng et al., 2000) and the cortical association of Miranda in *lgl aPKC* mutants (Lee et al., 2006b).

Results

To investigate the mechanism by which asymmetric aPKC is translated into polarized fate determinant segregation, we reconstituted Miranda cortical displacement by aPKC in cultured *Drosophila* S2 cells. We quantitatively assayed cortical-to-cytoplasmic signal ratios and found Miranda localizes robustly to the cortex of S2 cells (Fig. 5a,i; Fig. 6). The Miranda region responsible for cortical targeting in neuroblasts (residues 1-290)(Fuerstenberg et al., 1998; Matsuzaki et al., 1998; Shen et al., 1998) also localizes to the cell cortex (Fig. 5b,i) suggesting that the association mechanism is the same in both contexts. Expression of aPKC with Miranda leads to loss of cortical staining with a concomitant increase in cytoplasmic signal (Fig. 5c,i) and kinase activity is required for this effect, as a kinase-dead aPKC variant does not displace Miranda (Fig. 5d,i). We conclude that aPKC removes Miranda from the S2 cell cortex, and that this system can be used as a model for investigating aPKC-dependent Miranda cortical displacement.

As the S2 cell system accurately recapitulates aPKC-mediated cortical displacement of Miranda, we used it to test the requirements for Lgl and Myosin II in this process. We first tested whether Myosin II can displace Miranda from the cell cortex via aPKC. As cortical association of Myosin II has been proposed to exclude Miranda from

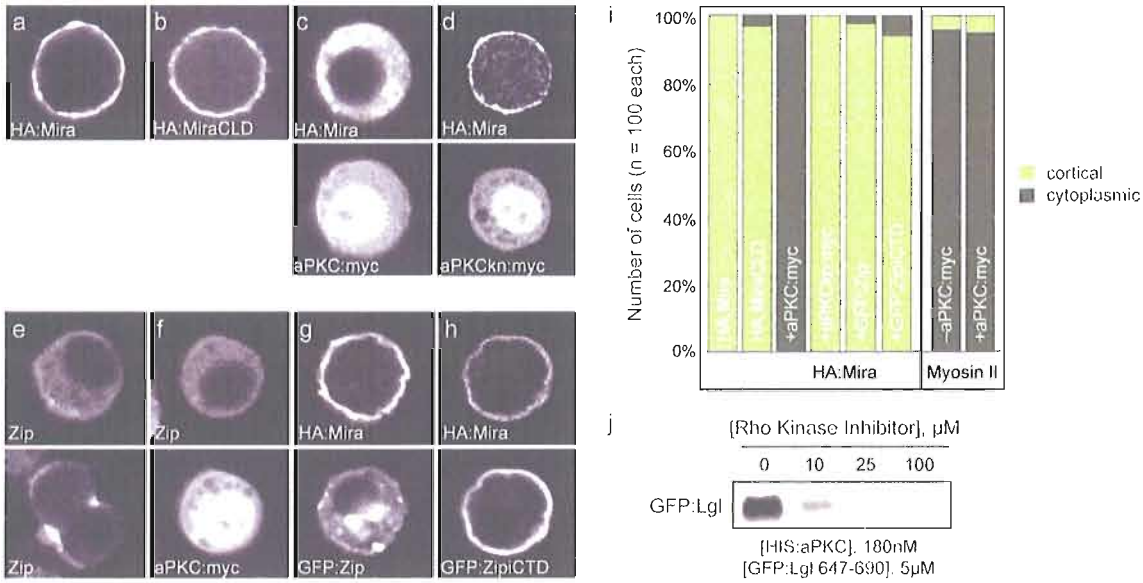


Figure 5. aPKC displaces Miranda from the cortex of S2 cells independently of Myosin II. (A-H) Expression of the various constructs in fixed S2 cells and stained by indicated markers; n of 100 cells each experiment. (A,B) HA:Mira (100%), and the cortical localization domain of Miranda (96%), shows cortical localization in S2 cells. (C) Miranda is displaced into the cytoplasm in the presence of aPKC:myc (100%), (D) but remains cortical in the presence of aPKCkn:myc (100%). (E) Myosin II is cytoplasmic and enriched at the cleavage furrow in the absence of aPKC:myc (96%) and (F) in the presence of aPKC:myc (94%). (G) Miranda remains cortical in the presence of GFP:Zipper (97%) and (H) in the presence of the interphase cortical targeting domain of GFP:Zipper (94%). (I) Quantification of Miranda and Myosin II localization in the indicated backgrounds. (J) aPKC activity is inhibited in the presence increasing concentrations of Rho kinase inhibitor.

the cortex (Barros et al., 2003), the level of cortical Myosin II is predicted to increase in cells expressing sufficient aPKC to displace Miranda. Endogenous Myosin II is cytoplasmic in S2 cells and enriches at the cleavage furrow during mitosis (Fig. 5e,i). However, we observe no increase in cortical Myosin II in cells expressing aPKC (Fig. 5f,i). To further test the role of Myosin II in Miranda cortical displacement, we over-expressed full-length heavy chain (Fig. 5g,i; Zipper in *Drosophila*) or a fragment that localizes predominantly to the cortex (Fig. 5h,i)(Liu et al., 2008), but still observed no

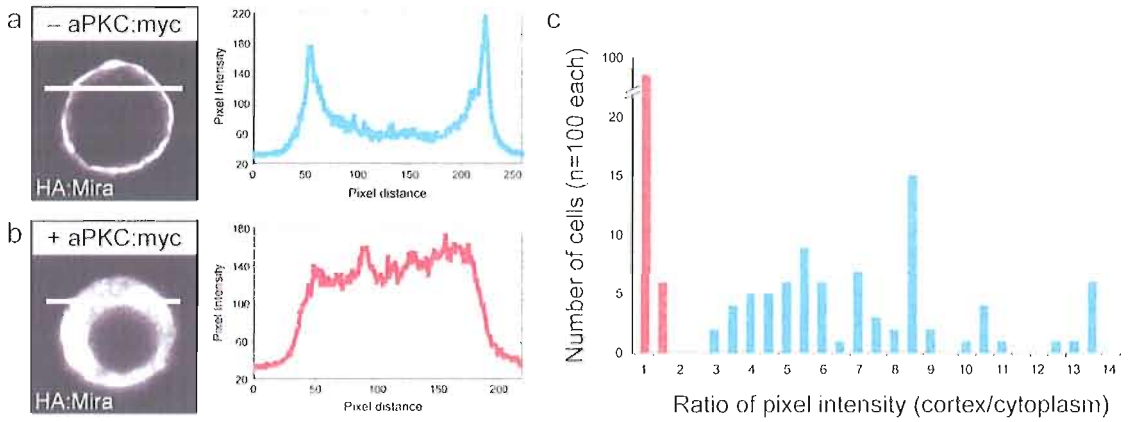


Figure 6. Quantification of S2 cell signal intensity. (A,B) HA:Mira is cortical in the absence of aPKC:myc and cytoplasmic in the presence of aPKC:myc. Plotting pixel intensities along a line generates a plot of pixel intensities over distance. (C) Plotting pixel intensity at the cortex to pixel intensity in the cytoplasm generates a histogram of cortical-to-cytoplasmic signal ratios over 100 cells from each background. Cells with ratios 1.5 or below were scored as cytoplasmic whereas cells with ratios 2 or more were scored as cortical.

effect on Miranda localization. Overexpression of Zipper in neuroblasts also has no effect on Miranda (Peng & Doe, personal communication). We conclude that cortical Myosin II is not sufficient to displace Miranda from the cortex.

The requirement for Myosin II in aPKC-mediated displacement of Miranda arises from the observation that Miranda localizes uniformly cortical in neuroblasts from embryos injected with the Rho kinase inhibitor Y-27632 (Barros et al., 2003; Erben et al., 2008). Rho kinase phosphorylates the regulatory light chain of myosin II, which is required for filament assembly and motor activity (Kosako et al., 2000; Matsumura, 2005) such that inhibition of this kinase leads to inactivation of Myosin II. As Miranda localization in Y-27632 treated neuroblasts is identical to *apkc* neuroblasts and kinase inhibitors are often not completely specific, we hypothesized that the inhibitor may also suppress aPKC activity. Using an *in vitro* kinase assay with purified, recombinant aPKC,

we find that Y-27632 efficiently inhibits aPKC with an $IC_{50} < 10$ μM (Fig. 5j). Given the cross-reactivity of Y-27632 with aPKC, the Miranda phenotype in embryos injected with this drug is likely the result from direct inhibition of aPKC kinase activity. Taken with the results from the S2 cortical displacement assay, we conclude Myosin II is not involved in mediating aPKC removal of Miranda from the cell cortex.

We next tested if Lgl is required for Miranda cortical exclusion. We utilized Lgl3A, a non-phosphorylatable version of Lgl, which induces uniformly cortical Miranda when expressed in neuroblasts (Betschinger et al., 2003). When Lgl3A is expressed with Miranda in S2 cells, Miranda and Lgl3A co-localize at the cortex as expected (Fig. 7a,c). However, when aPKC is also expressed, Lgl3A remains at the cortex but Miranda is displaced into the cytoplasm (Fig. 7b,c), inconsistent with Lgl phosphorylation being a prerequisite for Miranda displacement. In fact, neuroblasts from *lgl aPKC* neuroblasts exhibit uniformly cortical Miranda (Lee et al., 2006b), indicating that Lgl is not required for Miranda cortical association.

Lgl has been thought to play a role in aPKC-mediated displacement of Miranda based on the uniform cortical localization of Miranda in Lgl3A-expressing neuroblasts. To explore the nature of this phenotype more closely, we examined these neuroblasts and found their apical aPKC and Par-6 crescents to be severely reduced compared to wild-type (Fig. 7d-g). Based on this observation, we propose that the uniform cortical Miranda phenotype observed in Lgl3A expressing neuroblasts results from excessive inhibition of aPKC by Lgl3A relative to the wild-type protein, rather than arising from a requirement for Lgl phosphorylation in Miranda displacement. We tested this model by examining

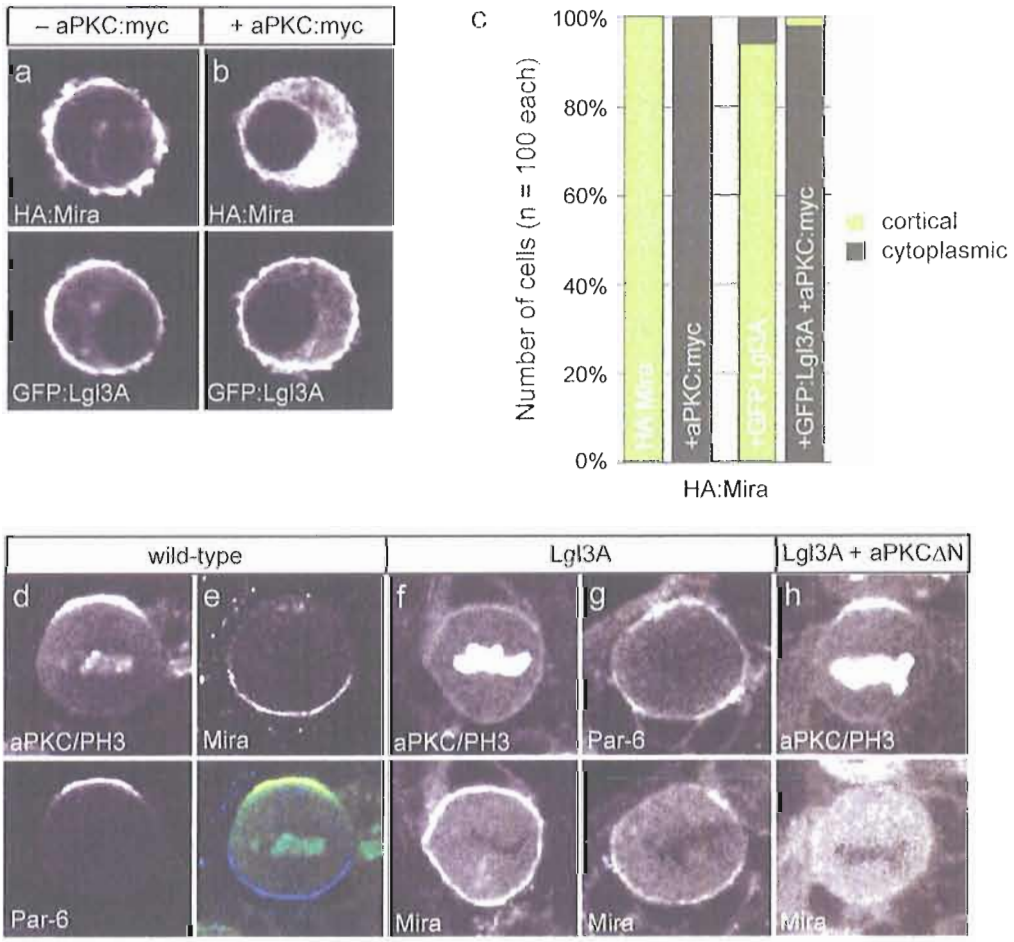


Figure 7. aPKC can displace Miranda from the cortex independently of Lgl. (A) Miranda is cortical in the presence of GFP:Lgl3A (94%; n=100) in fixed S2 cells as expected, (B) but is displaced into the cytoplasmic in the presence of GFP:Lgl3A and aPKC:myc (98%; n=100). (C) Quantification of Miranda localization in Lgl3A-expressing background. (D-H) Wild-type and expression of the indicated *UAS* transgenes by *worniu-GAL4* in brains at 96h after larval hatching (ALH) and labeled with the indicated markers. (D,E) Wild-type larval neuroblasts display normal cortical polarity, whereas (F) Lgl3A-expressing neuroblasts show disrupted apical aPKC and Par-6 crescents. (G) Cytoplasmic aPKCΔN can displace Mira from the cortex of neuroblasts even in the presence of Lgl3A.

whether an active, predominantly cytoplasmic aPKC (aPKCΔN) that no longer binds Lgl through Par-6 (N-terminal portion that binds Par-6 is missing)(Betschinger et al., 2003) could bypass the presence of Lgl3A and drive Miranda from the neuroblast cortex. If the

Lgl3A phenotype results from inhibition of aPKC activity, aPKCΔN should overcome this inhibition and displace Miranda from the cortex, whereas if phosphorylation of Lgl is required for Miranda displacement, Miranda should remain cortical. We find that Miranda is efficiently driven into the cytoplasm of neuroblasts expressing Lgl3A and aPKCΔN (Fig. 7h), further indicating that Lgl phosphorylation is not required for aPKC-mediated Miranda cortical displacement.

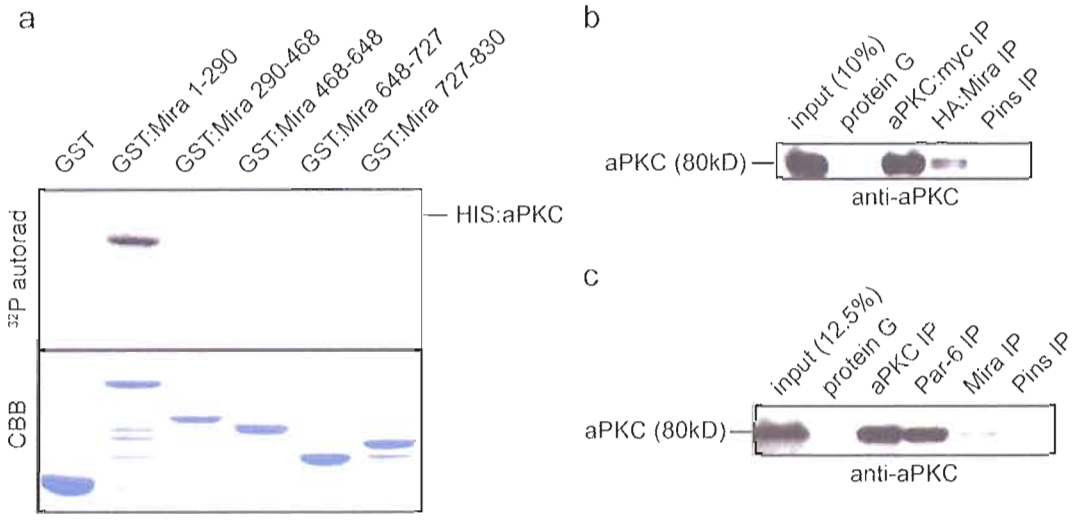


Figure 8. aPKC binds and phosphorylates Miranda. (A) Phosphor Image of GST and GST:Mira fusion proteins in the presence of recombinant aPKC and ^{32}P. GST:Mira 1-290 is phosphorylated whereas all other constructs are not. Commassie stain of gel as loading control. (B) Immunoblot of S2 cell lysates from cells transfected with aPKC:myc and HA:Mira immunoprecipitated with anti-aPKC, -HA, or -Pins antibodies. Protein G and anti-Pins antibody used as controls. (C) Immunoblot of embryonic lysate immunoprecipitated with anti-aPKC, -Par-6, -Mira, or -Pins antibodies. Controls as in (B).

Given that Lgl and Myosin II do not appear to be involved in Miranda displacement by aPKC, we hypothesized that aPKC may act directly on Miranda. To determine whether aPKC directly phosphorylates Miranda, we expressed and purified

several Miranda truncations and incubated them with recombinantly expressed and purified aPKC. We observed phosphorylation of Miranda 1-290, but not fragments that lack this region indicating that the phosphorylation site(s) occur within the Miranda cortical localization domain (Fig. 8a). Consistent with Miranda being an aPKC substrate, Miranda co-immunoprecipitates with aPKC from transfected S2 cells (Fig. 8b) and *Drosophila* embryonic extracts (Fig. 8c) indicating that the two proteins interact with one another *in vivo*. We identified the sites within Miranda that are phosphorylated by aPKC using mass spectrometry, which lead to the identification of several groups of phosphorylated residues (Fig. 9).

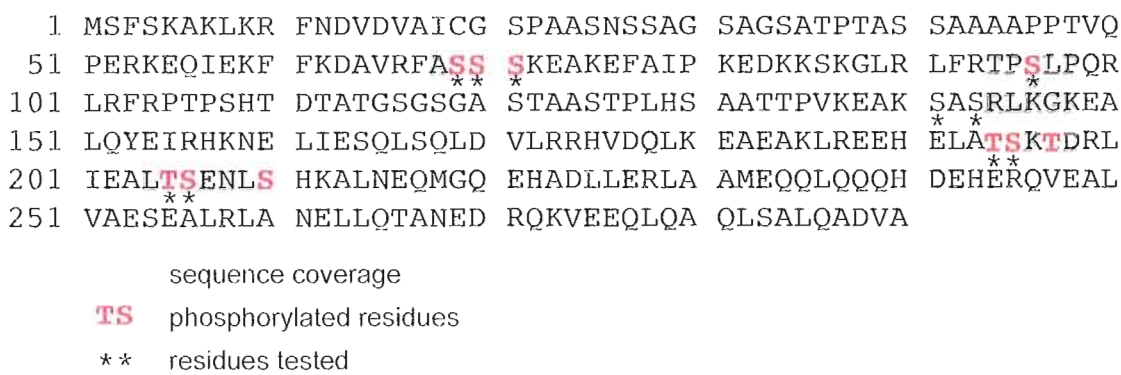


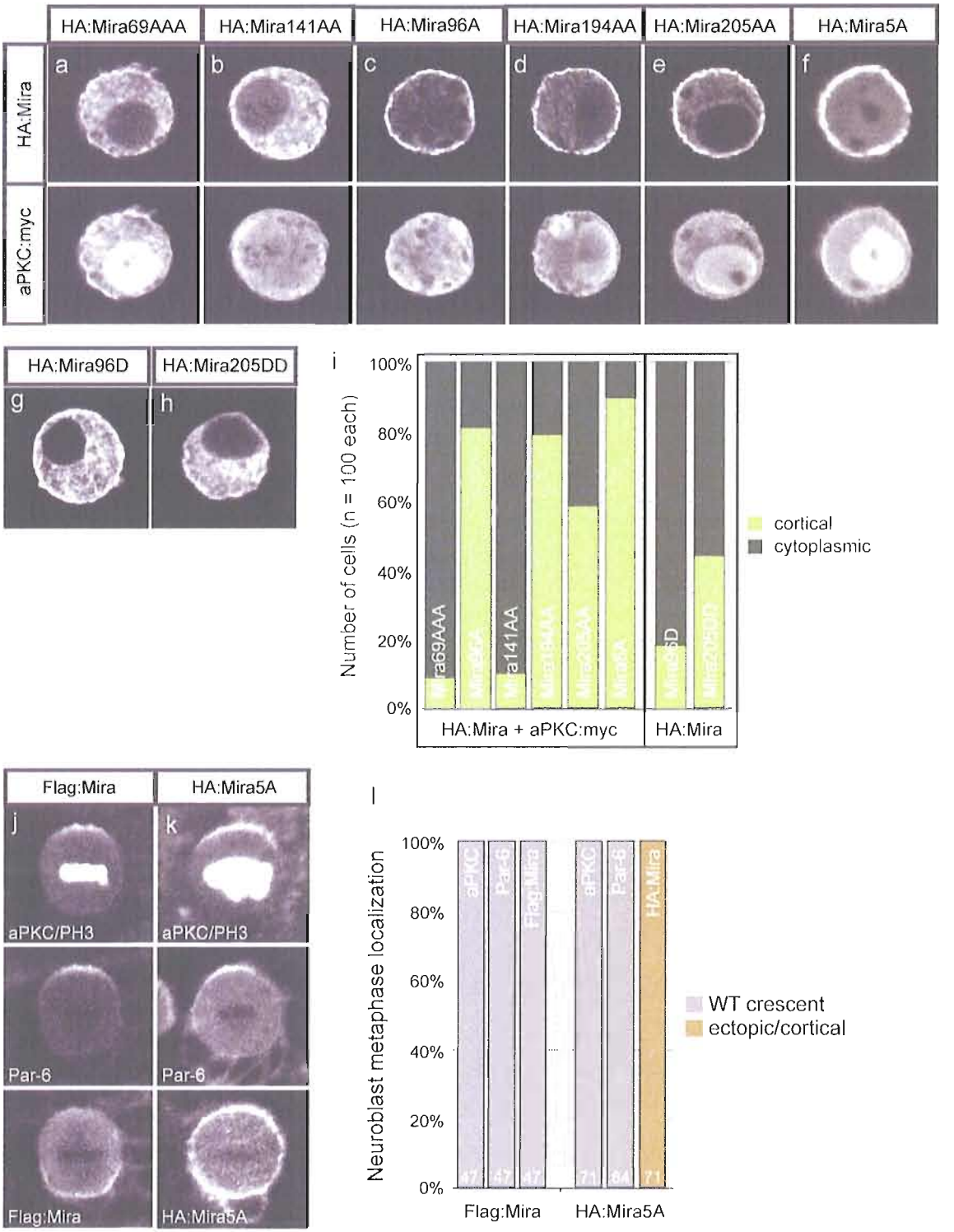
Figure 9. Mass spectrometry of phosphorylated Miranda 1-290. Sequence of Mira 1-290. Purple-highlighted residues indicates coverage by LC/MS/MS, red lettering indicates residues phosphorylated, and stars indicate residues tested by mutation to alanine. Phosphorylation prediction programs predicted residues 141 and 143 to be highly likely for phosphorylation by aPKC.

To determine which phosphorylation sites within Miranda may be responsible for cortical displacement, we mutated them to alanine and determined whether these proteins could be displaced into the cytoplasm by aPKC. In the S2 cortical targeting assay,

mutation of residues 69, 70, and 71 (Mira69AA) or 141 and 143 (Mira141AA) did not have any appreciable effect on Mira cortical exclusion in the presence of aPKC (Fig. 10a,b,i). However, mutation of residue 96 (Mira96A), residues 194 and 195 (Mira194AA), or residues 205 and 206 (Mira205AA) disrupted Miranda displacement by aPKC (Fig. 10c-e,i). We combined the mutated residues that reduced aPKC's ability to displace Miranda from the cortex into one protein (Mira5A) and assayed its localization in the presence of aPKC. Mira5A remains cortical in the presence of aPKC (90% cortical) indicating that these sites regulate cortical exclusion of Miranda (Fig. 10f,i). We conclude that aPKC phosphorylation of Miranda is necessary for excluding Miranda from the cell cortex of S2 cells.

We next tested whether phosphorylation alone can lead to Miranda displacement from the S2 cell cortex. We generated aspartic acid mutations at sites of aPKC phosphorylation and examined the localization of the phosphomimetic protein in the absence of aPKC. In the S2 cortical targeting assay, mutation of residue 96 (Mira96D) or residues 205 and 206 (Mira205DD) disrupted Miranda cortical targeting indicating phosphorylation is sufficient for cortical exclusion (Fig. 10g-i). The results of both alanine and aspartic acid mutations demonstrate that phosphorylation is necessary and sufficient for excluding Miranda from the cortex of S2 cells.

Figure 10. aPKC phosphorylation is necessary and sufficient to displace Miranda from the cortex. (A-H) Expression of HA:Mira constructs with or without aPKC:myc in fixed S2 cells and stained by indicated markers; n of 100 cells each experiment. (A) Miranda with alanine mutations in residues 69, 70, and 71 (Mira69AAA) localizes predominantly to the cytoplasm in the presence of aPKC:myc (92%), (B) as well as a construct with alanine mutations in residues 141 and 143 (Mira141AA; 90%). (C) Miranda with alanine mutations in residues 96 (Mira96A) is predominantly cortical in the presence of aPKC:myc (82%), (D) as well as constructs with alanine mutations in residues 194 and 195 (Mira194AA; 79%) or (E) in residues 205 and 206 (Mira205AA; 58%). (F) Miranda with alanine mutations in all five residues showing substantial effects (Mira5A; residues 96, 194, 195, 205, and 206) localizes predominantly to the cortex in the presence of aPKC:myc (90%). (G,H) Miranda with aspartic acid mutations at residues 96 or residues 205 and 206 are predominantly cytoplasmic in the absence of aPKC:myc (Mira96D- 82%; Mira205DD- 55%). (I) Quantification of Miranda point mutants in the presence, or absence, of aPKC:myc. (J,K) Expression of *UAS* transgenes by *pros-GAL4* in brains at 96 hALH and stained by the indicated markers. (J) Flag:Mira was used as a control and localized to the basal cortex (100%; n=47), whereas aPKC and Par-6 localized to the apical cortex (100%; n=47). (K) HA:Mira5A was not restricted to the basal cortex and localized uniformly cortical (100%; n=71), whereas aPKC (100%; n=71) and Par-6 (100%; n=64) remained wild-type. (L) Quantification of neuroblast polarity with the indicated transgenes.


HA:Mira69AAA
HA:Mira141AA
HA:Mira96A
HA:Mira194AA
HA:Mira205AA
HA:Mira5A
HA:Mira
aPKC:myc
a
b
c
d
e
f
HA:Mira96D
HA:Mira205DD
g
h
i
Number of cells (n = 100 each)
100%
80%
60%
40%
20%
0%
HA:Mira + aPKC:myc
HA:Mira
cortical
cytoplasmic
Flag:Mira
HA:Mira5A
j
k
aPKC/PH3
aPKC/PH3
Par-6
Par-6
Flag:Mira
HA:Mira5A
l
Neuroblast metaphase localization
100%
80%
60%
40%
20%
0%
aPKC
Par-6
Flag:Mira
aPKC
Par-6
HA:Mira
47
47
47
71
64
71
Flag:Mira
HA:Mira5A
WT crescent
ectopic/cortical

We verified that Miranda phosphorylation is required for cortical displacement in neuroblasts using transgenic flies expressing Mira5A. We expressed wild-type Miranda and Mira5A in neuroblasts using the *prospero-GAL4* driver, and assayed for Miranda localization and neuroblast polarity. Wild-type Miranda was properly restricted to the basal cortex throughout mitosis (Fig. 10j) as expected, whereas Mira5A was no longer excluded from the apical cortex and was uniformly cortical in 100% of neuroblasts (Fig. 4k). In both experiments Par-6 and aPKC apical polarity remained wild-type, as well as the localization of Numb, another cell fate-determinant (data not shown). We conclude that Miranda phosphorylation by aPKC excludes Miranda from the cortex of neuroblasts.

These results suggest that the mechanism of aPKC-mediated fate determinant segregation is much simpler than previously thought. Rather than utilizing a complicated cascade of negative interactions, direct phosphorylation by aPKC is sufficient to create mutually exclusive aPKC and fate determinant cortical domains. However, what is the function of Lgl if not to regulate Miranda cortical localization downstream of aPKC? Neuroblasts lacking *lgl* function exhibit ectopic aPKC and cytoplasmic or weakly basal Miranda (Lee et al., 2006b) indicating that Lgl acts upstream of aPKC in addition to being an aPKC substrate. To determine how Lgl might regulate aPKC, we assayed for additional neuroblast polarity defects in larval neuroblasts from *lgl* zygotic mutants. As previously described, these mutants exhibited ectopic aPKC and an increase in cytoplasmic Miranda, in stark contrast to wild-type neuroblasts (Fig. 11a-c). Likewise, we observed ectopic Par-6 that colocalized with aPKC suggesting that these proteins remain in complex (Fig. 11c). On the other hand, Baz localization was observed to be

unaffected by the loss of Lgl (Fig. 11d), indicating Lgl function is not required for the recruitment of this positive signal. Thus, we conclude that Lgl negatively regulates Par-6/aPKC activity at the basal cortex in a Baz-independent manner.

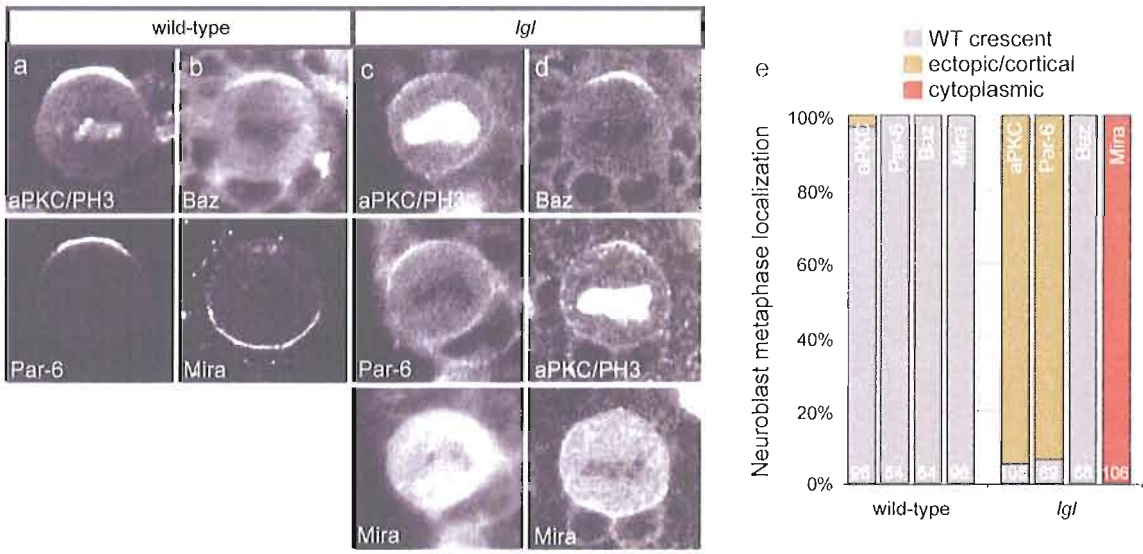


Figure 11. Lgl negatively regulates Par-6/aPKC activity at the basal cortex. (A-D) Brains at 96hALH and labeled with the indicated markers. (A,B) Wild-type neuroblasts display normal cortical polarity. (C,D) lgl^{334} neuroblasts display ectopic aPKC (95%; n=105) and Par-6 (94%; n=69) at metaphase, whereas Bazooka remained wild-type (100%; n=56). Miranda was cytoplasmically localized (100%; n=106).

Discussion

These results lead to a simplified mechanism for aPKC's role in neuroblast self-renewal and cell-fate segregation. Unlike previous models for cell-fate segregation that employ complicated multi-step inhibition involving aPKC, Lgl, Myosin II, and Miranda, we show aPKC directly displaces Miranda from the cortex, independent of Myosin II and Lgl. Rather than directly acting to mediate aPKC cell fate determinant asymmetry, the

tumor suppressor Lgl functions in segregating Miranda to the basal daughter cell by promoting asymmetric aPKC activity through inhibition of Par-6/aPKC at the basal cortex, independent of the positive signal Baz. The proposed role for Myosin II in this process may be artifactual, as we show that a chemical inhibitor of Rho kinase used in these studies efficiently inhibits aPKC, providing an alternative explanation of these results. This new model dramatically simplifies our understanding of how asymmetric aPKC activity, a characteristic of many polarized systems, is translated into the segregation of cell fate determinants.

Materials and methods

S2 cell culture and quantification

S2 cells were cultured using Schneider's medium (Sigma) containing 10% FBS. Constructs were cloned into pMT and transfected using Effectene (Qiagen). We generated alanine and aspartic acid point mutations by site-directed mutagenesis using pMT Mira as a template. In order to quantitate Mira localization, we analyzed 100 cells transfected with HA:Mira in the presence, or absence, of aPKC:myc in ImageJ to generate histograms based on pixel intensity versus pixel distance. We plotted the pixel intensity at the cortex versus the pixel intensity in the cytoplasm on a graph to generate a profile of each background to observe whether any overlap exists in our quantification (Fig. 6a-c). Any cell in which the ratio of cortex to cytoplasm staining was 1.5 or less we denoted as cytoplasmic. Likewise, any cell in which the ratio is 2 or more was denoted as cortical.

Fly strains

Oregon R (wild type), *lgl*^{334} (Bloomington), *worniu-GAL4*, *pros-GAL4, UAS-HA:Mira5A, UAS-aPKCΔN* (Betschinger et al., 2003), *UAS-Flag:Mira* (gift from C. Doe), *UAS-lgl3A* (Betschinger et al., 2003). Stocks were balanced over *CyO*; *CyO, actin::GFP*; *TM3, actin::GFP, Ser, e*; or *TM3, Sb.* To produce Mira5A transgenic animals, we PCR amplified and subcloned the coding sequence into the pUAST vector downstream of a 5' hemagglutinin (HA) tag and generated transformants using standard methods.

Antibodies and immunofluorescent staining

We fixed and stained larval brains and S2 cells as previously described (Siegrist and Doe, 2006). Wild-type and *lgl* mutant larvae were aged at 25˚C until 96 h after larval hatching (ALH). Flag:Mira and HA:Mira5A mutant larvae were aged at 30˚C until 96 h ALH (*pros-GAL4*). Lgl3A and aPKCΔN mutant were aged at 30˚C until 96 h ALH (*worniu-GAL4*). 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)(Atwood et al., 2007); rat anti-Mira (1:500)(Atwood et al., 2007); rabbit anti-Phospho-Histone H3 (1:1000; Upstate); guinea pig anti-Baz (1:1000)(Siller et al., 2006); mouse anti-HA (1:1000; Covance); mouse anti-Flag (1:100; Sigma); rabbit anti-zipper (1:2000)(Liu et al., 2008); rabbit anti-GFP (1:1000; Torrey Pines); and rat anti-Pins (1:100)(Nipper et al., 2007). 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.

Protein purification, binding experiments, and mass spectrometry

All proteins were expressed and purified as previously described (Atwood et al., 2007). *Drosophila* embryonic lysate was prepared as previously described (Atwood et al., 2007). We immunoprecipitated using approximately 5 μg rabbit anti-aPKC, rat anti-Par-6, rat anti-Pins, or mouse anti-HA as per manufacturer's protocol (GE Healthcare). To determine which immunoprecipitates contained aPKC, we separated samples by SDS-PAGE and transferred to nitrocellulose followed by probing with anti-aPKC (1:2000) antibody.

For mass spectrometry, SDS-PAGE gel of phosphorylated Mira 1-290 was stained with coomassie and gel slices containing protein was digested with trypsin and resuspended in formic acid. Samples were analyzed using OrbiTrap with neurtral loss for +2, +3, +4 ions. Spectra were analyzed using MASCOT and X!Tandem.

Kinase assay

We incubated purified HIS:aPKC (Atwood et al., 2007) at 30°C for 15 min in reaction buffer (20mM HEPES pH 7.5, 10mM $MgCl_2$, 1mM DTT, 10mM ATP) then added GST or GST:Mira fragments (10μM final concentration) and 17nM [g-^{32}P]-labeled ATP for 20 additional min. We quenched the reaction by addition of SDS loading buffer and heating at 95°C for 5 min and determined the extent of phosphorylation by SDS-

PAGE and exposure onto a Phosphor screen (Molecular Dynamics) and detected using STORM 860.

Bridge to Chapter IV

Chapter III discussed how aPKC is restricted to the apical neuroblast cortex and how aPKC activity translates into cell-fate determinant segregation. Lgl restricts Par-6/aPKC to the apical cortex through mutual inhibition. aPKC segregates Miranda to the basal cortex by phosphorylating and displacing Miranda from the apical cortex. This process is independent of Myosin II and Lgl. Chapter IV will discuss how Par-6 and Cdc42 regulate aPKC kinase activity to segregate cell-fate determinants.

CHAPTER IV

PAR-6 AND CDC42 REGULATE APKC KINASE ACTIVITY THROUGH PSEUDOSUBSTRATE ALLOSTERY

Introduction

Atypical Protein Kinase C (aPKC) is essential for many cellular functions, such as cell polarity, and is conserved across metazoa. *Drosophila* neural stem cells (neuroblasts) utilizes aPKC's role in cellular polarity to asymmetrically divide and generate a large, apical self-renewing neuroblast and a small, basal ganglion mother cell that divides once to form two neurons or glia. aPKC is in a complex with Par-6 and both are recruited to the neuroblast apical cortex through an interaction with the Rho GTPase Cdc42 (Atwood et al., 2007). aPKC phosphorylates many cellular substrates including the cell-fate determinants Miranda (Mira) and Numb, which are excluded from the neuroblast apical cortex and segregate basally [unpublished results; (Smith et al., 2007)]. Par-6 suppresses aPKC kinase activity and Cdc42 partially relieves this repression (Atwood et al., 2007), but the change in kinase activity from repressed to activated is only two-fold begging the question of whether the neuroblast operates under such a small dynamic range.

Apical localization of aPKC kinase activity is critical for its function in neuroblasts as cytoplasmic or uniformly cortical aPKC have defects in cell-fate

determinant segregation (Lee et al., 2006b), but little is known about how aPKC is regulated. aPKC has several conserved domains that bind regulatory factors to modulate kinase activity. The regulatory region contains an amino-terminal PB1 domain that interacts with Par-6 (Noda et al., 2003), a psuedosubstrate (PS) motif, and a variant cysteine-rich region that binds acidic phospholipids such as PIP3 (Nakanishi et al., 1993). A hinge-region attaches the regulatory region to the catalytic domain and a PKC-specific V5 domain at the carboxy-terminus. The catalytic domain of aPKC is initially activated by PDK1 phosphorylation in the activation loop (Le Good et al., 1998) and subsequent autophosphorylation in the hydrophobic motif (Standaert et al., 2001). Many factors directly bind and alter aPKC kinase activity such as Cdc42/Par-6 (Atwood et al., 2007), Dishevelled (Zhang et al., 2007), Dap160 (Chabu and Doe, 2008), Par-4 (prostate androgen response-4), LIP (lambda-interacting protein), src, and several lipid components (Suzuki et al., 2003). Unraveling the mechanism of how aPKC responds to its many inputs would give insight into its diverse biological functions.

aPKC has several unique characteristics that suggest it's kinase activity might be differentially regulated from other members of the PKC family. aPKC's catalytic domain has several deviations that result in a decrease in sensitivity to several inhibitors acting on the nucleotide-binding site (Spitaler et al., 2000). aPKC is the only known nucleotide-binding protein that has an alanine in place of glycine at position six in the glycine-rich loop, and lysine to arginine mutations in the nucleotide-binding site that abolishes kinase activity of all other PKCs has no effect on aPKC. All members of the PKC family are autoinhibited by their PS in their basal state, but surprisingly, aPKC seems to have a high

level of basal activity that is repressed by Par-6 *in vitro* (Atwood et al., 2007) suggesting the PS motif may be playing a different role in aPKC than previously thought. How aPKC kinase activity is altered and what role specific domains play remains unclear.

Here we examined the mechanism of regulation of aPKC kinase activity. Repressed aPKC targeted to the cortex by Par-6 that is not activated by Cdc42 disrupts neuroblast polarity and results in cortical Mira. aPKC undergoes an intramolecular interaction between the V5 domain and the regulatory region that is perturbed upon Par-6 binding. Par-6 promotes interaction between the catalytic domain and the PS motif to inhibit kinase activity. Cdc42 induces an intermolecular interaction between the V5 domain and Par-6 PDZ to disrupt the PS interaction and partially rescue activity. The V5-PDZ interaction is necessary for activation as disruption of this interaction *in vitro* and *in vivo* abolishes Cdc42 activation and results in neuroblast polarity defects. We conclude that the neuroblast operates under a small aPKC dynamic range and that range is mediated by intra- and intermolecular interactions involving aPKC and its regulatory partners.

Results

Cdc42 activation of aPKC is necessary for neuroblast polarity

Cdc42 partially activates a repressed Par-6/aPKC complex (Atwood et al., 2007), but it is unknown whether this small dynamic range is sufficient to generate robust neuroblast polarity. To investigate how a Par-6/aPKC complex that cannot be activated by Cdc42 functions, we constructed a Par-6 mutant that localizes to the cortex of

neuroblasts in the absence of Cdc42 binding. We tagged Par-6, and a Par-6ISAA mutant that abolishes Cdc42 binding (Atwood et al., 2007), with a C-terminal CAAX motif that allows insertion into membranes. We expressed the Par-6 mutants specifically in neuroblasts with the *pros-Gal4* driver and assayed for polarity defects. HA:Par-6caax

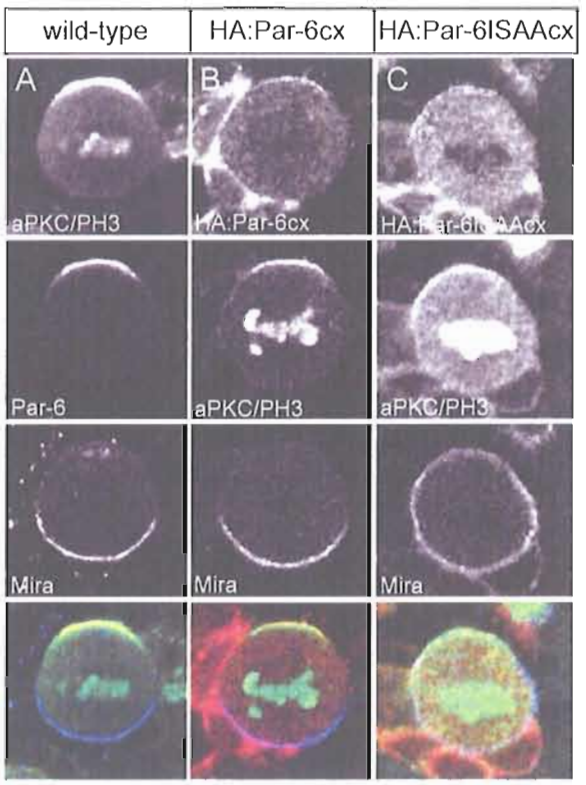


Figure 12. Cdc42 is necessary to activate aPKC at the cortex. (A-C) Brains at 96hALH and labeled with the indicated markers. (A) Wild-type neuroblasts display normal cortical polarity. (B) HA:Par-6caax expressed in mitotic neuroblasts using *prosGAL4* display apical localization, whereas aPKC and Miranda are wild-type. (C) Expression of HA:Par-6ISAAcaax using *prosGAL4* display weakly cortical and high cytoplasmic localization. aPKC is apically-enriched with ectopic cortical localization and high cytoplasmic staining and Mirands is uniformly cortical.

localized to the apical cortex of dividing neuroblasts with weak ectopic staining (Fig. 12B). aPKC was apically enriched with weak ectopic staining whereas Mira localization remained wild-type suggesting the ectopic aPKC is not active, presumably because no Cdc42 is present in the complex. HA:Par-6ISAAcaax localized mainly to the cytoplasm with weak cortical staining (Fig. 12C). aPKC was apically enriched with weak ectopic staining, however, Mira was uniformly cortical suggesting Par-6ISAAcaax acts in a dominant fashion to repress endogenous aPKC activation. Par-6ISAA is cytoplasmic with no ectopic localization and does not show any neuroblast polarity defects (Atwood et al., 2007) indicating that Cdc42 activation at the cortex is necessary to establish basal polarity.

V5 domain interacts with the regulatory region of aPKC

In order to illuminate the conformational changes that aPKC undergoes in the presence of regulatory factors, we looked for the presence of intramolecular interactions within aPKC that could be altered upon binding of Par-6/Cdc42. The crystal structure of the catalytic domain of PKCiota revealed that the V5 domain, which contains the autophosphorylation region, participates in an intramolecular interaction with the amino-terminal lobe of the kinase domain (Messerschmidt et al., 2005). The V5 domain has also been shown to interact with the regulatory regions in PKCbeta and PKCepsilon (Kheifets and Mochly-Rosen, 2007). As such, we used yeast two-hybrid as an assay to detect an intramolecular interaction between the V5 and regulatory regions of aPKC. All constructs containing the PB1 domain and PS motif interacted with V5, with a construct only

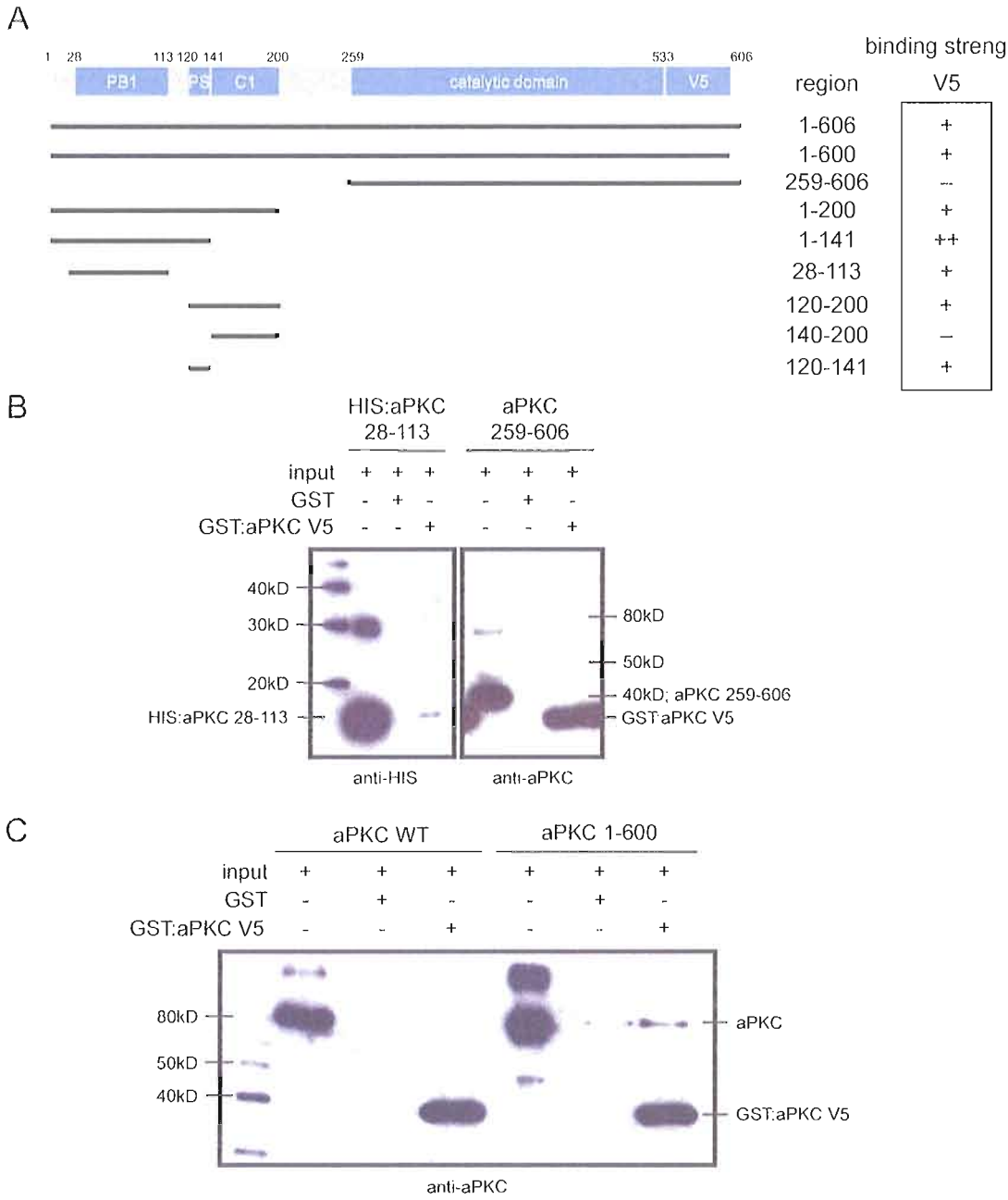


Figure 13. aPKC V5 interacts with the regulatory region. (A) Yeast two-hybrid assay shows interaction with all contructs containing the PB1 domain (28-113) and the PS motif (120-141). A more robust interaction is observed when both the PB1 and PS domains are expressed together. (B) Western blot showing GST:aPKC V5 is able to bind aPKC PB1 but not the catalytic domain (259-606) *in vitro*. (C) Western blot showing GST:aPKC V5 is able to bind an aPKC with the intramolecular interaction disrupted (1-600) but not an aPKC where the intramolecular interaction is intact (wild-type).

containing both domains interacting more robustly (Fig. 13A). The V5/regulatory region interaction occurs *in vitro* as GST:V5 bound both GFP:PB1 and GFP:PS, but not the kinase domain (Fig. 13B and data not shown). In fact, the intramolecular interaction occurs in full-length aPKC as GST:V5 bound aPKC 1-600 – where the intramolecular interaction should be perturbed – but did not bind full-length aPKC – where the intramolecular interaction is preserved (Fig. 13C). Interestingly, interaction between the PS motif and V5 domain would preclude autoinhibition of aPKC as PS could not interact with the catalytic domain.

We next decided to explore how the regulatory region of aPKC affects kinase activity by using variants of aPKC with amino-terminal truncations (Fig. 14A). We tested the activity of these aPKC variants by assaying how well they phosphorylate the endogenous substrate Lgl. Full-length and the catalytic domain of aPKC showed similar activities (Fig. 14B,C) suggesting that aPKC is catalytically active it its basal state, unlike other PKC family members. Deletion of the PB1 domain, or PB1 and PS motif of aPKC results in a slight increase in kinase activity. However, deletion through the C1 domain of aPKC resulted in a three-fold decrease in kinase activity suggesting the hinge region has repressive interactions that are rescued by addition of the regulatory region (Fig. 14B,C). A small deletion at the C-terminus of aPKC that partially disrupts the intramolecular interaction did not affect activity (Fig. 14B,C). We next assayed whether disruption of the PS motif affected kinase activity. aPKC's PS motif is 100% identical to its mammalian homologues, so we mutated four arginine residues flanking the alanine that sits in the active site to alanines. This mutant displays a small decrease in kinase activity compared

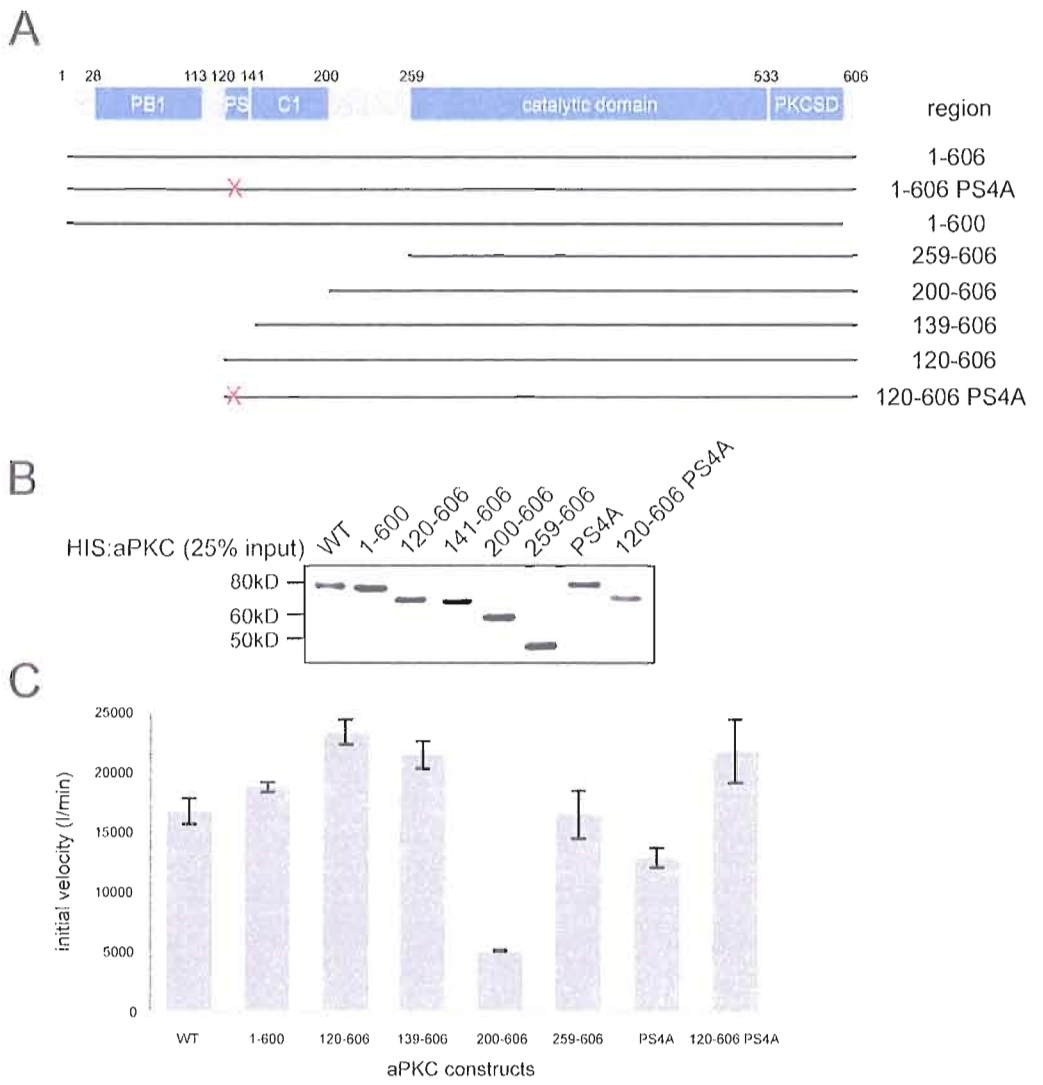


Figure 14. The regulatory region of aPKC regulates its kinase activity. (A) Schematic of aPKC contructs used in kinase assay. (B) Western blot showing concentrations of various aPKCs used in kinase assay. (C) Catalytic domain of aPKC is as active as wild-type protein. N-terminal domains (PB1/PS) partially repress aPKC activity. Hinge region connecting the catalytic domain and regulatory regions contain repressive interactions that are relieved upon addition of the regulatory region. PS point mutations slightly decrease kinase activity compared to wild-type.

to wild-type. The same PS point mutants were added to aPKC 120-606 (PB1 deletion) and no substantial difference was seen compared to aPKC 120-606 (Fig. 14B,C) indicating the PS motif does not play a role in inhibiting aPKC basal activity. All aPKC constructs were active *in vivo* as expression in S2 cells were sufficient to displace Lgl from the cortex (data not shown), which is an *in vivo* assay for aPKC kinase activity (Betschinger et al., 2003). We conclude that there are both activating and inhibiting interactions within aPKC that may be utilized when regulatory factors bind, and that aPKC basal activity has constitutively high activity that is not repressed as its PKC counterparts.

Par-6 promotes PS-mediated inhibition of aPKC

How Par-6 inhibits aPKC activity is unknown. One possibility is that Par-6 completely disrupts the aPKC intramolecular interaction. This could promote PS inhibition of aPKC by blocking sequestration of the PS motif by V5. In order to determine whether Par-6 disrupts aPKC's intramolecular interaction, we bound GST:V5 to aPKC 1-600 and asked whether Par-6 could disrupt this interaction. Par-6 blocked GST:V5 from binding aPKC 1-600 (Fig. 15A) indicating Par-6 binding disrupts the aPKC intramolecular interaction. We next assayed whether the PS motif plays a role in Par-6 inhibition. We added the PS motif in *trans* to aPKC and aPKC/Par-6 to assay its affect on kinase activity. As expected, the PS motif inhibited kinase activity in both contexts suggesting that the PS could play a role in Par-6 inhibition (Fig. 15B). We mutated the central alanine in the PS motif that sits in the catalytic pocket to aspartic acid, which has been shown to reduce its affinity to the catalytic site and abolish

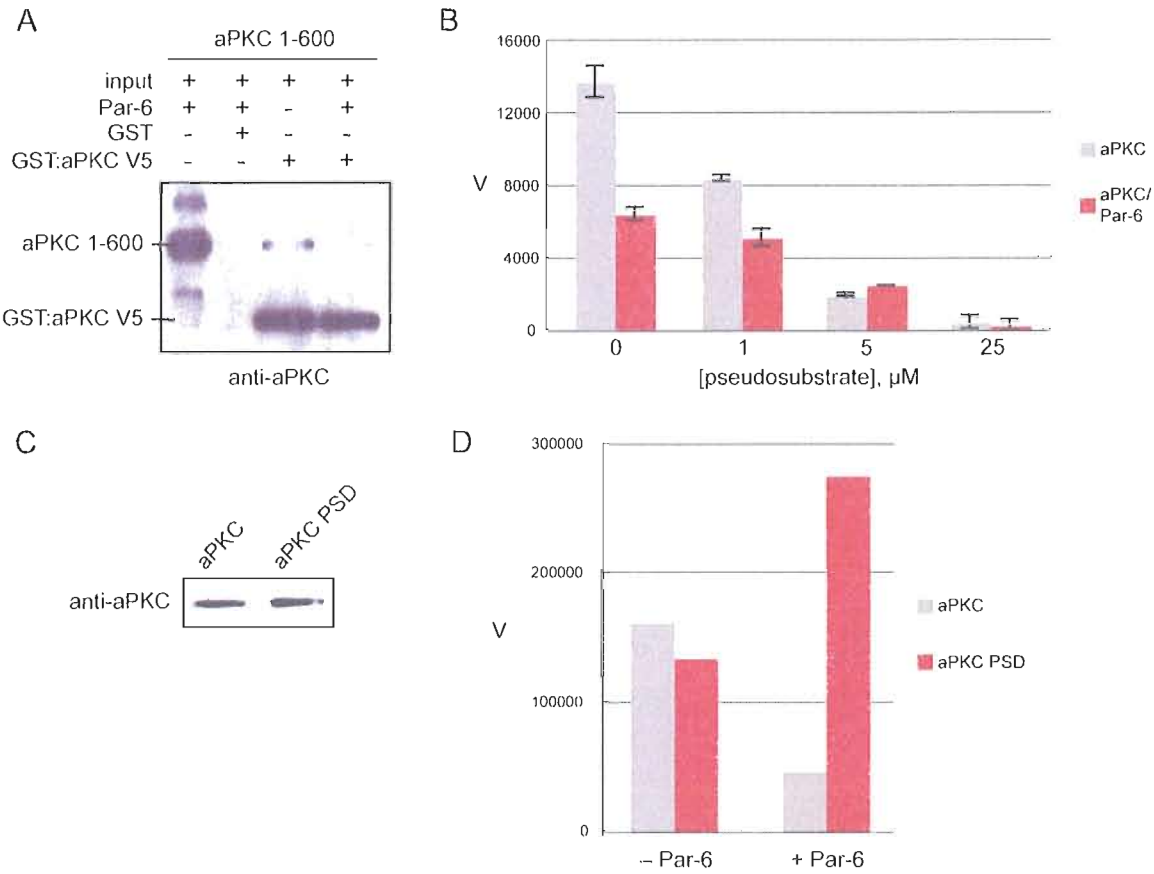


Figure 15. Par-6 promotes PS-mediated inhibition of aPKC. (A) Western blot of GST:aPKC V5 binding aPKC 1-600 in the absence, but not the presence, of Par-6. (B) PS peptide inhibits both aPKC and Par-6/aPKC in a dose-dependent manner. Inhibition of Par-6/aPKC is delayed at low concentrations of PS peptide. (C) Western blot of aPKC proteins used in kinase assay. (D) Par-6 can no longer repress an aPKC where the PS motif is disrupted by replacement of the central alanine with aspartic acid (aPKC PSD). Activity of aPKC PSD increases upon addition of Par-6, suggesting other inhibitory interactions within aPKC exist apart from the PS motif.

autoinhibition of PKC (Pears et al., 1991). Mutation at this site does not significantly perturb aPKC kinase activity (Fig. 15C,D). However, Par-6 no longer inhibits aPKC, but causes an increase in activity suggesting other inhibitory interactions exist within aPKC independent of the PS motif that Par-6 is able to relieve. We conclude that Par-6

promotes PS-mediated inhibition of the catalytic domain of aPKC by inhibiting the PS/V5 interaction.

Cdc42 promotes binding between Par-6 PDZ and aPKC V5

According to our model, Par-6 inhibits aPKC kinase activity by promoting the PS/catalytic domain interaction. As Cdc42 partially relieves Par-6 repression of aPKC, Cdc42 may partially disrupt the PS/catalytic domain interaction resulting in improved catalytic activity. One way Cdc42 could do this is by inducing the PDZ domain of Par-6 to bind the V5 domain of aPKC. Cdc42 is known to induce Par-6 binding to C-terminal PDZ ligands in other systems (Peterson et al., 2004) and alignment of the C-terminus of aPKC with its human homologues and with PKCα reveals several conserved residues including a C-terminal valine characteristic of C-terminal PDZ ligands (Fig. 16A). Whereas PKCα is a class one PDZ ligand, both human and *Drosophila* aPKCs are class three PDZ ligands (-D/E/K/R-X-ϕ-COOH; where ϕ = hydrophobic residue). To test whether aPKC is a PDZ ligand for Par-6, we performed a pull-down using GST:V5, which includes the PDZ ligand, and the Par6 CRIB/PDZ domain. Par-6 CRIB/PDZ is not sufficient to bind GST:V5, however, upon addition of Cdc42•GTPγS, Par-6 CRIB/PDZ was able to bind GST:V5 indicating that Cdc42 promotes Par-6 PDZ binding to the C-terminal aPKC PDZ ligand within V5 (Fig. 16B). This binding event also occurs *in vivo* as GST:Par-6 CRIB/PDZ incubated with *Drosophila* embryonic lysate supplemented with Cdc42•GTPγS is able to bind endogenous aPKC (Fig. 16C). A strong prediction of our model is that Par-6 PDZ ligands will have the ability to disrupt Cdc42 activation of

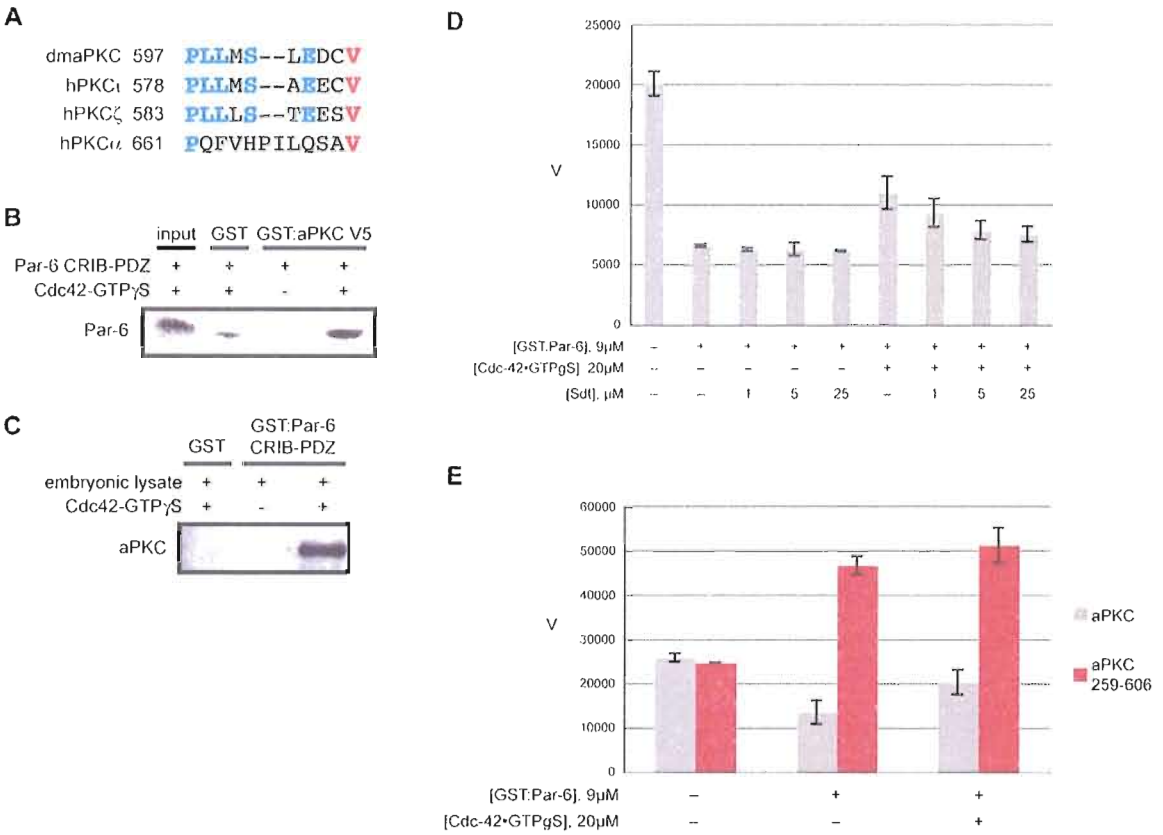


Figure 16. Cdc42 activates aPKC by promoting Par-6 PDZ interaction with aPKC V5. (A) Alignment of aPKC with human aPKCs and PKCalpha. (B) Western blot of GST:aPKC V5 interacting with the Par-6 CRIB/PDZ domain in a Cdc42-dependent manner. (C) Western blot of GST:Par-6 CRIB/PDZ interacting with endogenous aPKC from embryonic lysate in a Cdc42-dependent manner. (D) Cdc42 activation of aPKC is inhibited upon addition of Stardust peptide in a dose-dependent manner. Par-6 repressed aPKC is not repressed further by Sdt. (E) Addition of Par-6 and Cdc42 can enhance kinase activity of the catalytic domain of aPKC when the regulatory region is absent.

aPKC by competing with the Par-6 PDZ/aPKC V5 interaction. In fact, a Sdt peptide that binds Par-6 PDZ (Penkert et al., 2004) also inhibits Cdc42-activated aPKC but not Par-6-inhibited aPKC (Fig. 16D). Interestingly, addition of Par-6 and Cdc42 to the catalytic domain of aPKC induces a two-fold increase in aPKC activity, suggesting the catalytic domain may have some additional inhibitory interactions that are relieved upon binding

of Par-6 to the V5 domain of aPKC (Fig. 16E). In fact, Dishevelled has been shown to bind the V5 domain of aPKC and induce an increase in aPKC kinase activity (Zhang et al., 2007).

aPKC V5 and Par-6 PDZ interaction is necessary for aPKC activity in neuroblasts

We next tested whether the Par-6 PDZ/aPKC V5 interaction is important for its function in neuroblast polarity. We expressed HA:aPKC and HA:aPKC 1-600 specifically in neuroblasts using the *prosGal4* promoter. HA:aPKC localized apically in dividing neuroblasts and had no effect on neuroblast polarity as endogenous aPKC, Par-6, and Mira showed wild-type localization (Fig. 17A). HA:aPKC 1-600 localized weakly to the apical cortex with cytoplasmic staining in dividing neuroblasts (Fig. 17B). Endogenous Par-6 showed the same localization as aPKC 1-600, while Baz localization remained wild-type (Fig. 17C). However, Mira localization was substantially disrupted and localized uniformly cortical indicating aPKC 1-600 activity is inhibited and acts in a dominant-negative fashion (Fig. 17B). We conclude that Cdc42-dependent Par-6 PDZ/aPKC V5 interaction is necessary for robust aPKC activity and neuroblast polarity.

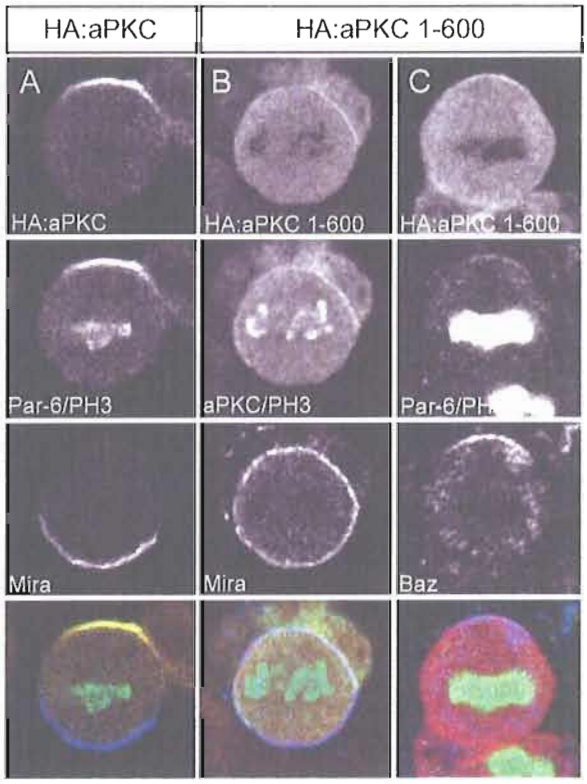


Figure 17. aPKC V5 and Par-6 PDZ interaction is necessary for kinase activity at the cortex. (A-C) Brains at 96hALH, *UAS* transgenes expressed using *prosGAL4*, and labeled with the indicated markers. (A) HA:aPKC localizes to the apical cortex and no defects are observed in Par-6 or Miranda localization. (B,C) HA:aPKC 1-600 localizes weakly to the apical cortex and is highly cytoplasmic. Par-6 is apical with reduced crescents and Miranda is uniformly cortical. Bazooka remains wild-type.

Discussion

Despite the importance of aPKC in the development of organisms, little is know about how it responds to regulatory factors. Here we show how negative (Par-6) and positive (Cdc42) regulatory factors alter aPKC kinase activity. aPKC is highly active in its basal state and undergoes an intramolecular interaction that sequesters its PS motif from the catalytic domain. Par-6 suppresses aPKC by disrupting aPKC's intramolecular interaction thereby promoting PS binding to the catalytic domain, whereas Cdc42

partially activates aPKC by disrupting PS-depedent inhibition through induction of Par-6 PDZ/aPKC V5 interaction. The small increase in activity from a Par-6-repressed state to a Cdc42-activated one is sufficient in promoting neuroblast polarity, revealing an amazing response of *Drosophila* neuroblasts to a small range in signal.

All other PKC family members are thought to be autoinhibited in their basal state by interaction of their PS motif to the substrate-binding pocket of the catalytic domain. Surprisingly, we show that the PS motif in aPKC does not autoinhibit aPKC but is sequestered away by an intramolecular interaction with the V5 domain. Par-6 disrupts this interaction to promote inhibition by the PS motif. This suggests other PB1-binding proteins would also inhibit aPKC kinase activity, such as p62/ZIP or MEK5 (Diaz-Meco and Moscat, 2001; Moscat and Diaz-Meco, 2000), and other aPKC inhibitors such as Par-4 would act through a similar mechanism. Cdc42 sequesters the PS away from the substrate-binding pocket by promoting an interaction between Par-6 PDZ and aPKC V5, suggesting another mechanism to inhibit aPKC activity. Any Par-6 PDZ ligand, such as Crumbs (Lemmers et al., 2004), Pals1/Stardust (Hurd et al., 2003), Par-3/Bazooka (Joberty et al., 2000), or EphrinB1 (Lee et al., 2008), that competes with Cdc42 or binds the PDZ domain in the presence of Cdc42 would inhibit aPKC by restoring PS binding to the catalytic domain. Likewise, any interaction that disrupts the PS/catalytic domain interaction would activate aPKC. Recently, Dishevelled (Zhang et al., 2007) has been shown to activate aPKC by binding the C-terminus of aPKC. Dap160 (Chabu and Doe, 2008) also activates aPKC, presumably through a similar mechanism.

Cdc42 activates aPKC roughly two-fold over Par-6 repression (Atwood et al., 2007). Surprisingly, this small activation is sufficient to polarize the neuroblast as cortical Par-6 that cannot bind Cdc42 or a localized aPKC that cannot be activated by Cdc42 are unable to segregate Miranda to the basal cortex. This implies that the neuroblast is ultrasensitive to aPKC kinase activity as low activity cannot elicit a physiological response, but once a threshold is reached (within the two-fold Cdc42-activated range), the neuroblast is competent to respond to this signal.

Materials and methods

S2 cell culture

S2 cells were cultured using Schneider's medium (Sigma) containing 10% FBS. Constructs were cloned into pMT and transfected using Effectene (Qiagen). Cells were quantified by assaying cortical-to-cytoplasmic ratios. Cells with ratios of 1.5 or less were scored as cytoplasmic whereas cells with ratios of 2 or more were scored as cortical.

Fly strains

Oregon R (wild type), *worniu-GAL4, pros-GAL4, UAS-HA:Par-6caax, UAS-HA:Par-6ISAA* (Atwood et al., 2007), *UAS-HA:Par-6ISAAcaax*, *UAS-HA:aPKC*, *UAS-HA:aPKC 1-600*. Stocks were balanced over *CyO* or *TM3, Sb.* To produce Par-6caax and Par-6ISAAcaax transgenic animals, we PCR amplified and subcloned the coding sequence with TGCAAATTCTTA at the 3' end into the pUAST vector downstream of a 5' hemagglutinin (HA) tag and generated transformants using standard methods. To

produce aPKC and aPKC 1-600 trangenic animals, we PCR amplified and subcloned the coding sequence into the pUAST vector downstream of a 5' hemagglutinin (HA) tag and generated transformants using standard methods.

Antibodies and immunofluorescent staining

We fixed and stained larval brains and S2 cells as previously described (Siegrist and Doe, 2006). Wild-type and UAS expressed larvae (*pros-GAL4*) were aged at 25°C until 96 h after larval hatching (ALH). 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)(Atwood et al., 2007); rat anti-Mira (1:500)(Atwood et al., 2007); rabbit anti-Phospho-Histone H3 (1:1000; Upstate); guinea pig anti-Baz (1:1000)(Siller et al., 2006); mouse anti-HA (1:1000; Covance). 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.

Protein purification and binding experiments

All proteins were expressed and purified as previously described (Atwood et al., 2007). *Drosophila* embryonic lysate was prepared as previously described (Atwood et al., 2007). Yeast two-hybrid assays were performed by cloning constructs into pGBK and pGAD plasmids and transfecting into yeast strain AH109. Yeast were plated onto SD(-

LEU)(-TRP) and SD(-HIS)(-LEU)(-TRP) plates, incubated at 30˚C for 96 hours, and assayed for growth.

Kinase assay

We incubated purified HIS:aPKC (Atwood et al., 2007) at 30˚C for 15 min in reaction buffer (20mM HEPES pH 7.5, 10mM $MgCl_2$, 1mM DTT, 10mM ATP) then added GFP:Lgl 647-690 (5μM final concentration) and 17nM [g-^{32}P]-labeled ATP for the indicated amounts of time. We quenched the reaction by addition of SDS loading buffer and heating at 95˚C for 5 min and determined the extent of phosphorylation by SDS-PAGE and exposure onto a Phosphor screen (Molecular Dynamics) and detected using STORM 860.

Bridge to Chapter V

Chapter IV discussed how aPKC kinase activity is regulated by Par-6 and Cdc42. aPKC undergoes an intramolecular interaction that provides high basal activty. Par-6 disrupts this intramolecular interaction and promotes PS-mediated inhibition. Cdc42 promotes an additional interaction between Par-6 and aPKC to disrupted PS-mediated inhibition and activate aPKC kinase activity. Chapter V will go on to discuss how cortical actin regulates cortical polarity and aPKC kinase activity.

CHAPTER V

CDC42 REGULATES ACTIN DYNAMICS AND POLARITY AT THE NEUROBLAST CORTEX THROUGH MBT AND COFILIN

Introduction

Drosophila neural stem cells (neuroblasts) establish apical and basal polarity early in mitosis to orient the mitotic spindle and generate a self-renewed neuroblast and a ganglion mother cell that will typically divide once to form two neurons or glia. Cortical actin is critical in this process as drug-induced disruption results in defects in apical and basal polarity components (Broadus and Doe, 1997). Rho GTPases such as Cdc42 are well-known regulators of the actin cytoskeleton (Etienne-Manneville and Hall, 2002) and Cdc42 has recently been implicated in recruitment of Par complex members Par-6 and aPKC, but not Par-3 (Bazooka), to the apical neuroblast cortex where they function to restrict the cell-fate determinant Miranda to the basal cortex (Atwood et al., 2007). How Cdc42 regulates cortical actin to establish and maintain polarity within the neuroblast remains unknown.

Cdc42 has many downstream effectors that control cell polarity and cytoskeletal components (Etienne-Manneville, 2004). Cdc42 has been shown to regulate cofilin, an actin effector, during establishment of neuronal polarity (Garvalov et al., 2007). Cofilin

enhances the turnover of actin by severing or nucleating actin filaments, depending on its concentration (Andrianantoandro and Pollard, 2006), and is inhibited by phosphorylation (Moriyama et al., 1996). *Drosophila* cofilin, or Twinstar (Tsr), is required for cell division (Gunsalus et al., 1995), motility (Chen et al., 2001), morphology (Menzel et al., 2007), and polarity (Blair et al., 2006; Garvalov et al., 2007). Despite its important role, little is known about how Tsr regulation of actin translates into cortical polarity.

Cdc42 also binds and recruits p21-activated kinase (PAK) to regulate actin-dependent processes (Zhao and Manser, 2005). *Drosophila* Mushroom bodies tiny (Mbt) is a group 2 PAK kinase that similar to mammalian PAK4-6 and is localized, but not regulated by, Cdc42 (Hofmann et al., 2004). *mbt* mutants have fewer neurons in the brain (Melzig et al., 1998) and photoreceptor cells in the ommatidium (Schneeberger and Raabe, 2003) suggesting a role in cell proliferation and differentiation. Tsr is phosphorylated by Mbt and overexpression of constitutively active Mbt results in severe defects in actin organization and adherens junction formation (Menzel et al., 2007). Whether Cdc42 regulates cotical actin though Mbt and Tsr is unknown.

Here we show that Mbt localizes to the apical cortex of dividing neuroblasts downstream of Cdc42. Mbt regulates neuroblast polarity as *mbt* neuroblasts have defects in aPKC, Par-6, and Miranda, but not Bazooka, localization suggesting cortical actin can regulate cortical polarity. Accordingly, loss of actin polymerization in Latrunculin A-treated neuroblasts have similar defects in cortical polarity. *tsr* neuroblasts also have defects in cortical polarity suggesting a mechanism whereby Cdc42 recruits Mbt to the

apical cortex to inactivate Tsr and establish stable actin-rich cortical domains that allow robust recruitment of apical polarity members.

Results

Mbt localizes to the apical cortex of neuroblasts downstream of Cdc42

Active Cdc42 localizes to the apical cortex of *Drosophila* neuroblasts to recruit Par-6/aPKC and establish cortical polarity (Atwood et al., 2007). In an attempt to determine whether cortical actin also plays a role in establishing and maintaining cortical polarity, we assayed for the localization of a known downstream Cdc42 effector, Mbt. As expected, aPKC localizes to the apical cortex opposite of Miranda (Fig. 18A). Mbt colocalizes with aPKC at the apical cortex of dividing larval neuroblasts throughout mitosis suggesting a possible role in regulating apical polarity (Fig. 18B-E). Mbt is known to directly bind Cdc42 through its N-terminal CRIB domain (Schneeberger and Raabe, 2003). Accordingly, Mbt apical localization is lost in *cdc42* neuroblasts (Fig. 18G), but is wild-type in *par6* neuroblasts (Fig. 18F), indicating Mbt localizes to the apical cortex through Cdc42 in a Par-6-independent manner.

mbt *neuroblasts have defects in cortical polarity*

We next assayed whether mbt neuroblasts have any defects in cortical polarity. We used the $mbt^{EY08341}$ allele that contains a P-element insertion in the 5' UTR, 27bp downstream from the start of the gene (Fig. 19A). These animals are homozygous viable with no detectable Mbt protein observed at the apical cortex of dividing larval

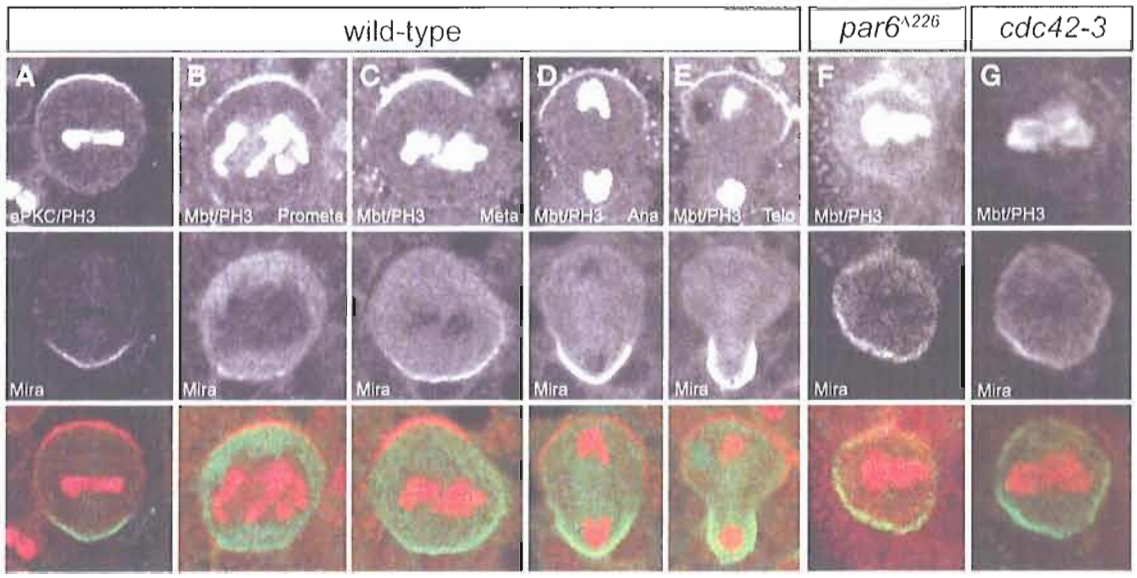


Figure 18. Mbt is apically localized downstream of Cdc42. (A-E) Wild-type brains at 96hALH and stained with the indicated markers. (A) aPKC and Miranda show wild-type localization. (B-E) Mbt localizes to the apical cortex throughout mitosis. (F) Mbt is still present at the apical cortex in *par6*$^{\Delta 226}$ neuroblasts. (G) Mbt localization is disrupted in *cdc42-3* neuroblasts.

neuroblasts (Fig. 19B). *mbt* neuroblasts have defects in aPKC (17%, n=90) and Par-6 (20%, n=50) with both protein showing ectopic cortical localization and reduced apical crescents (Fig. 19C,D,F). Miranda defects are more penetrant (38%, n=90) with the protein showing ectopic cortical localization with reduced basal crescents suggesting that the mislocalized Par-6/aPKC is inactive and cannot displace Mira from the cortex (Fig. 19C-F). Bazooka, however, remains wild-type (100%, n=20; Fig. 19E,F). These defects suggest Mbt is necessary for maintaining separate cortical polarity domains and activation of Par-6/aPKC, but not Bazooka, and alluding to a role for actin in maintaining cortical polarity as Mbt regulates actin-dependent processes (Menzel et al., 2007; Schneeberger and Raabe, 2003).

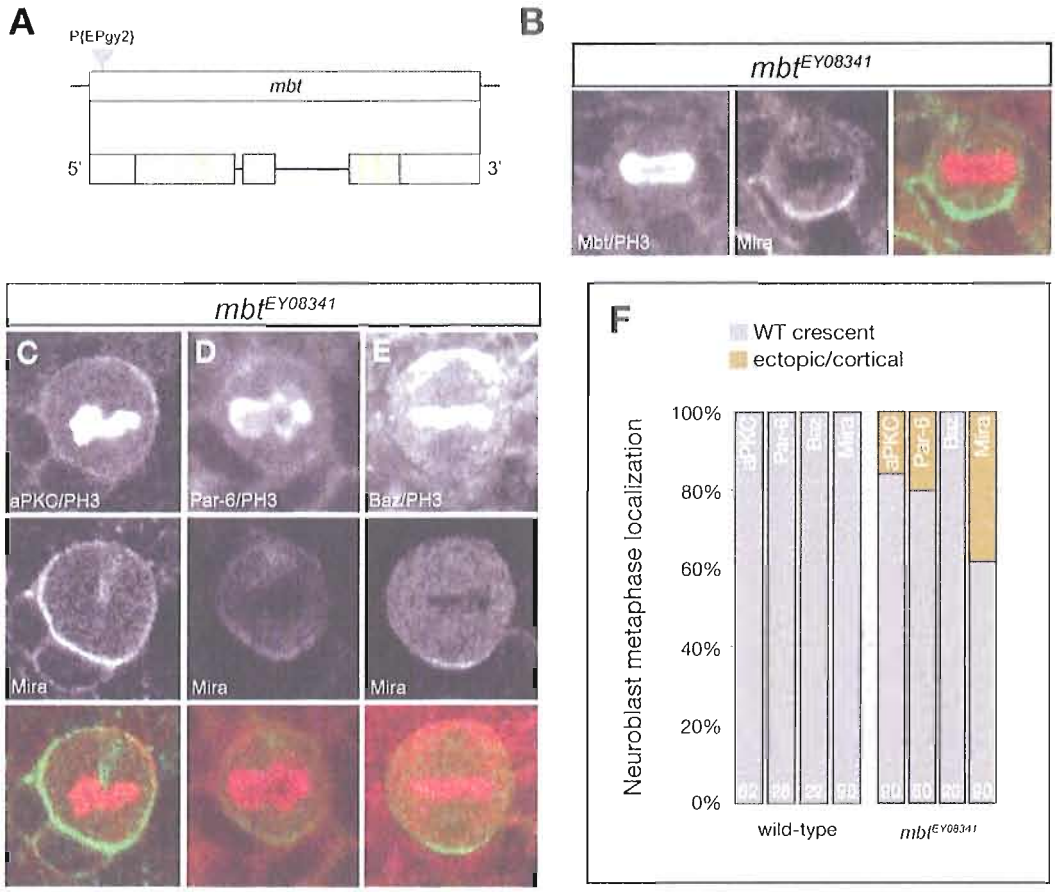


Figure 19. *mbt* mutants show defects in cortical polarity. (A) Schematic of mbt locus. Gray depicts untranslated regions and green depicts translated regions. P{EPgy2} inserted 27bp downstream from start of gene. (B) $mbt^{EY08341}$ neuroblasts have no detectable apical localization of Mbt. (C-E) Brains at 96hALH. aPKC, Par-6, and Miranda are ectopic cortical with reduced apical and basal crescents. (F) Quantification of *mbt* neuroblast polarity defects.

tsr *restricts aPKC to the apical cortex*

Mbt phosphorylates and inactivates Tsr (Menzel et al., 2007), suggesting a mechanism by which cortical actin is regulated by Cdc42 recruiting Mbt, resulting in inactivation of Tsr and promotion of stable actin at the apical cortex which serves as anchors for apical polarity components. As such, *tsr* mutants should display cortical polarity defects in dividing larval neuroblasts. We generated tsr^{N96A} neuroblast clones

using the MARCM system and assayed for cortical polarity defects. *tsr* neuroblats showed ectopic cortical aPKC with a concomitant increase in cytoplasmic Miranda (43%, n=7) suggesting stable actin promotes apical polarity at the neuroblast cortex (Fig. 20).

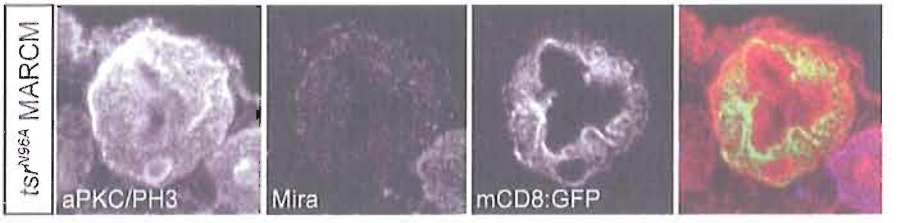


Figure 20. Tsr functions to restrict aPKC to the apical cortex. MARCM clones of tsr^{N96A} neuroblasts at 96hALH. aPKC is localized ectopically around the cortex. aPKC is still active as Miranda is displaced into the cytoplasm (43%, n=7). mCD8:GFP used to mark mutant clones.

Cortical actin functions to maintain cortical polarity

Drug-induced actin inhibition of primary neuroblast cultures result in defects in Inscuteable, an apical component, and Prospero and Staufen, two cargoes of Miranda (Broadus and Doe, 1997). In order to further explore how actin affects cortical polarity, we assayed for additional polarity defects in primary neuroblasts treated with actin and microtubule destabilizers. Primary neuroblasts with no drug treatment show the expected apical Par-6/aPKC and basal Miranda (Fig. 21A,B). Addition of the actin depolymerizer Latrunculin A results in ectopic cortical localization of Par-6 and aPKC whereas Miranda is delocalized into the cytoplasm suggesting apical polarity components can still localize to the cortex, but are unable to stay restricted to a specific cortical domain (Fig. 21C,D). When neuroblasts are treated with Nocadazole, which disrupts microtubules, cortical polarity remains wild-type suggesting microtubules do not play a role in maintaining

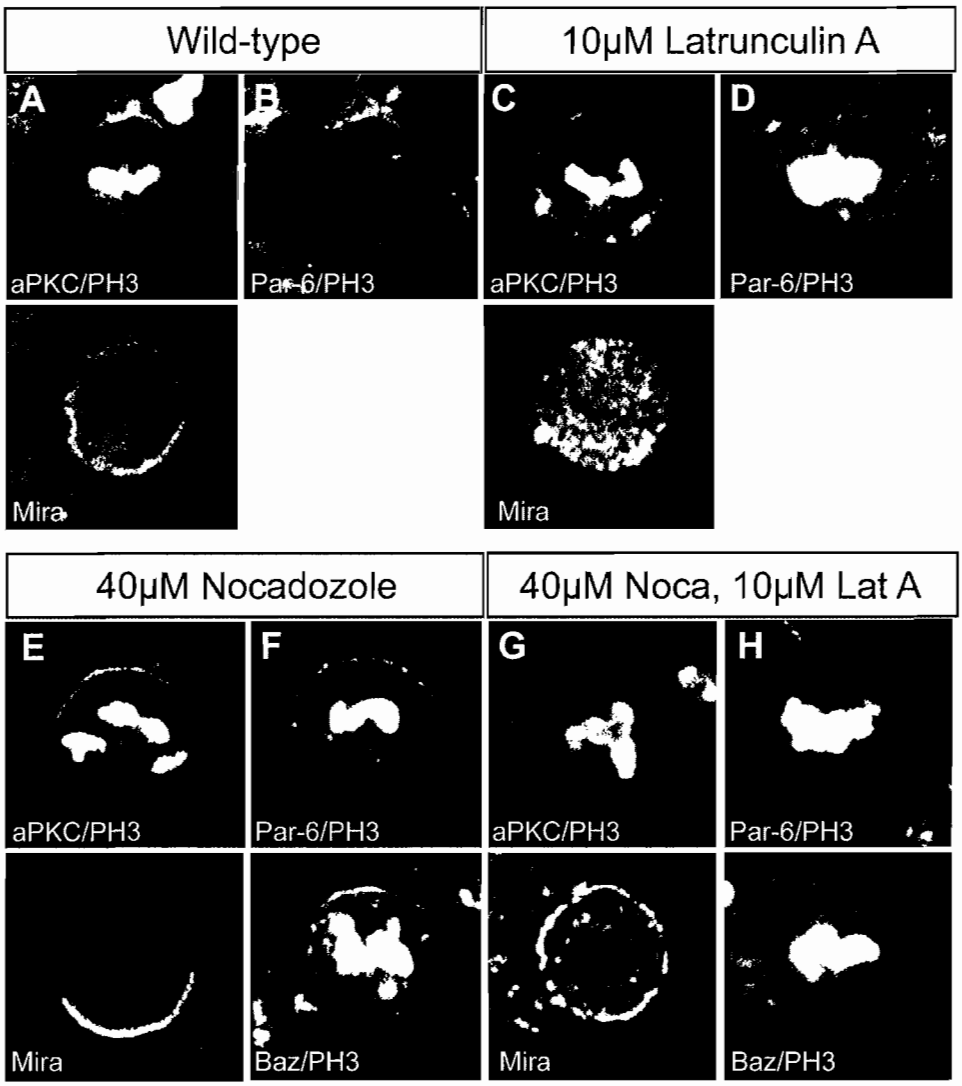


Figure 21. Actin promotes Par-6/aPKC localization and activity at the cortex. (A-H) Primary neuroblast cultures treated with the indicated drugs and stained with the indicated markers. (A,B) aPKC, Par-6, and Miranda crescents are wild-type. (C,D) Latrunculin A-treated neuroblasts show ectopical cortical localization of aPKC and Par6. aPKC is active as Miranda is displaced from the cortex. (E,F) Nocadozole-treated neuroblasts show no defects in cortical polarity and aPKC, Par-6, Bazooka, and Miranda remain wild-type. (G,H) Treatment of neuroblasts with both Nocadozole and Latrunculin A show weakly cortical and highly cytoplasmic aPKC with cytoplasmic Par-6 and Bazooka. aPKC is not active as Miranda is uniformly cortical and not displaced into the cytoplasm.

polarity (Fig. 21E,F). However, when both Nocadazole and Latrunculin A are used, cortical polarity is not established and Bazooka, Par-6 and aPKC are mostly cytoplasmic, whereas Miranda localizes uniformly around the cortex (Fig 21G,H) indicating aPKC is no longer active and cannot displace Miranda from the cortex. These experiments suggest microtubules function to establish cortical polarity whereas actin maintains apical polarity, in line with defects observed in *tsr* neuroblasts.

Discussion

How Cdc42 regulates cortical actin to maintain neuroblast polarity is unknown. We show that Cdc42 recruits Mbt to promote and activate apical polarity and maintain separate apical and basal domains. *mbt* neuroblasts show defects in restricting and activating apical polarity and results in defective Miranda localization. Mbt likely functions through Tsr as Mbt phosphorylates and inactivates the protein (Menzel et al., 2007), and *tsr* mutants show excessive aPKC/Par-6 recruitment to the cortex of neuroblasts that results in cytoplasmic Miranda. Additionally, disrupting actin using Latrunculin A mimics *tsr* polarity defects. We propose a simple mechanism whereby Cdc42 regulates actin and establishes separate cortical domains though recruitment of Mbt and inactivation of Tsr. This allows apical polarity components downstream of Bazooka to stay restricted to the apical cortex and allows for robust Par-6/aPKC activity.

Although cortical polarity is still established through microtubule-dependent processes, apical and basal domains are no longer separated. Interestingly, Mbt seems to function as an activator of aPKC activity as cortical aPKC in *mbt* neuroblasts is not

sufficient to displace Miranda from the cortex. Tsr seems to have the opposite effect as *tsr* neuroblasts show cortical aPKC is active and can displace Miranda to the cytoplasm. This suggests Tsr/cofilin acts as a tumor suppressor in neuroblasts, although its function is masked by failure of cells to undergo cytokinesis. In fact, Tsr/cofilin has been implicated in several tumor-related processes indicating an essential function of actin in maintaining distinct cortical domains in many cell types (Sidani et al., 2007; Wang et al., 2007b).

Materials and methods

Fly strains

Oregon R (wild type), $mbt^{EY08341}$ (Bloomington), tsr^{N96A} (Bloomington). Stocks were balanced over *CyO*. MARCM clones generated by crossing tsr^{N96A}, *FRTG13* to *hsFLP; TubGal80, FRTG13; TubGal4, UAS-mCD8GFP/TM6, Sb* (genereous gift from Melissa Rolls).

Antibodies and immunofluorescent staining

We fixed and stained larval brains as previously described (Siegrist and Doe, 2006). Wild-type and *mbt* larvae were aged at 25°C until 96 h after larval hatching (ALH). MARCM clones of tsr neuroblasts were generated by heat-shock at 37°C for 90 min and recovery at 25°C until dissection at 96 hALH. 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)(Atwood et al., 2007); rat anti-Mira (1:500)(Atwood

et al., 2007); rabbit anti-Phospho-Histone H3 (1:1000; Upstate); guinea pig anti-Baz (1:1000)(Siller et al., 2006); mouse anti-GFP (Upstate). 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.

Primary neuroblast culture

Primary neuroblasts were generated as previously described (Siegrist and Doe, 2006). Briefly, stages 9-10 embryos were homogenized in Chan and Gehrings's with 2% FBS media by seven passes through a dounce, passed through a sterile embryo mesh, and centrifuged at 1,000 *x g* for 5 min. Cells were washed 2x with media and plated onto a coverslip, allowed to settle for 30 min, and incubated with fresh media. Nocadazole was used at 40μM for 2 h, and Latrunculin A was used at 10μM for 20 min before fixing with 4% paraformaldehyde for 10 min.

CHAPTER VI

CONCLUDING REMARKS

Cell polarity is used in epithelial barrier functions, cell-to-cell contacts, cellular motility, and stem cell self-renewal and differentiation. Stem cells use polarity to choose between self-renewal and differentiation, and to avoid tumorigenesis or early quiescence. *Drosophila* neuroblasts asymmetrically divide to populate the fly central nervous system and must decide between stem cell self-renewal and differentiation through asymmetrically polarizing fate determinants. aPKC is a polarized protein that establishes apical and basal polarity and drives both neuroblast self-renewal and cell-fate. Localization and activity of aPKC is essential for its function and this work explores the mechanisms underlying these processes.

Neuroblast polarity is initially established by Baz (Kuchinke, 1998), which recruits many proteins to the apical cortex to establish polarity and orient the mitotic spindle. Chapter two shows how Baz recruits Par-6 and aPKC to the neuroblast apical cortex through asymmetric activation of Cdc42, leading to polarization of cortical aPKC kinase activity that is essential for directing neuroblast cell polarity, ACD, and sibling cell fate (Atwood, 2007). Cdc42 also recruits Par-6 and aPKC in many cell types, ranging from *C. elegans* embryonic blastomeres to mammalian epithelia (Aceto et al., 2006;

Joberty et al., 2000; Lin et al., 2000; Schonegg and Hyman, 2006), and seems to be a conserved process throughout all organisms that use Par proteins. Baz, Par-6, and aPKC have been considered to be part of a single complex (the Par complex). However, when Cdc42 function is perturbed, Par-6 and aPKC localization is disrupted and Baz remains unaffected suggesting Baz is only transiently associated with the Par-6/aPKC complex (Atwood et al., 2007) consistent with the observation that Baz does not colocalize with Par-6/aPKC in *Drosophila* embryonic epithelia (Harris and Peifer, 2005). In fact, Baz is a substrate for aPKC and phosphorylation of Baz is essential for its function in establishing epithelial junctions (Nagai-Tamai et al., 2002). *par6* mutants show reduced Cdc42 localization suggesting phosphorylation of Baz may also function to increase asymmetric Cdc42 activation, perhaps by increased GEF association, thereby reinforcing cell polarity (Atwood et al., 2007). 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). Baz is also known to bind GDP exchange factors (GEFs) (Zhang and Macara, 2006) which may induce accumulation of activated Cdc42 at the apical cortex.

Once aPKC is recruited to the neuroblast apical cortex, one of its functions is to direct the segregation of the cell-fate determinants Pros and Brat though Miranda, and Numb (Betschinger et al., 2003; Smith et al., 2007). Numb is asymmetrically localized through phosphorylation by aPKC, which dissociates Numb from the apical cell cortex leading to its segregation to the basal cortex (Smith et al., 2007). However, a complex model has been proposed to explain how aPKC activity leads to segregation of Miranda

(Barros et al., 2003). aPKC phosphorylates Lgl (Betschinger et al., 2003), which inhibits its ability to repress Myosin II (Barros et al., 2003). Myosin II has been proposed to physically displace Miranda from the cortex, "pushing" it from the apical to basal cortex (Barros et al., 2003). This leads to a complex pathway in which aPKC phosphorylates Lgl, preventing its inhibition of Myosin II, ultimately removing Miranda from the cortex at sites of aPKC activity. However, several key observations are inconsistent with this model, including the normal polarity observed in *zip* mutants (Peng et al., 2000) and the cortical association of Miranda in *lgl aPKC* mutants (Lee et al., 2006b). Chapter three shows a simplified mechanism for aPKC's role in neuroblast self-renewal and cell-fate segregation. Unlike the previous model, aPKC directly phosphorylates and displaces Miranda from the cortex, independent of Myosin II and Lgl. Myosin II's role in this process seems to be artifactual as a chemical inhibitor of Rho kinase used in previous studies efficiently inhibits aPKC, providing an alternative explanation of past results and serving as a reminder that inhibitors are notoriously promiscuous and conclusions based on these experiments alone should be analyzed with caution. Analogously, Lgl was thought to promote Miranda recruitment to the neuroblast cortex as overexpression of nonphosphorylateable Lgl3A results in uniformly cortical Miranda. Lgl3A has been used in many systems to analyze Lgl function such as oocyte polarity (Tian and Deng, 2008), sensory organ precursors (Langevin et al., 2005), and epithelia (Hutterer et al., 2004). However, Lgl3A also competitively inhibits cortical aPKC activity. When cytoplasmic aPKC is coexpressed with Lgl3A, cortical Miranda defects are rescued. Instead of promoting Miranda cortical recruitment, Lgl promotes asymmetric aPKC activity through

inhibition of Par-6/aPKC at the basal cortex, independent of Baz. Lgl's proposed role in many cell types may have to be reexamined in light of these findings.

aPKC's localization and activty has to be finely tuned for its function. *apkc* zygotic mutant animals die in early larval stages and its neuroblasts have defects in cortical polarity (Rolls et al., 2003). Targeting aPKC activity to the neuroblast cortex, but not inactive aPKC, results in defects in cell-fate segregation, massive neuroblast overproliferation, and tumors (Lee et al., 2006b). In fact, aPKC is considered an oncogene as overexpression of active aPKC can induce tumors in a variety of tissues (Kojima et al., 2008; Li et al., 2008; Regala et al., 2005). Chapter four shows how negative and positive regulatory factors alter aPKC kinase activity. aPKC has high basal activity that is repressed upon interaction with Par-6, whereas Cdc42 partially relieves this repression (Atwood et al., 2007). All other PKC family members are autoinhibited in their basal state by their PS motif. Interestingly, aPKC undergoes an intramolecular interaction between its V5 domain and the N-terminal regulatory region that sequesters its PS motif from the catalytic domain, resulting in high basal activity. Par-6 suppresses aPKC by disrupting aPKC's intramolecular interaction thereby promoting PS binding to the catalytic domain. This elegant rearrangement of domain interactions should be a universal mechanism for aPKC inhibition and any aPKC PB1-binding protein would inhibit aPKC kinase activity, such as p62/ZIP or MEK5 (Diaz-Meco and Moscat, 2001; Moscat and Diaz-Meco, 2000). Likewise, any interaction that disrupts the PS/catalytic domain interaction would activate aPKC, such as Dishevelled (Zhang et al., 2007) and Dap160 (Chabu and Doe, 2008). Cdc42 partially activates aPKC by interacting with Par-

6 and increasing the affinity of Par-6 PDZ domain to the V5 domain of aPKC, thus disrupting PS-mediated inhibition. Again, this should be a universal mechanism to activate a repressed Par-6/aPKC complex and any Par-6 PDZ ligand, such as Crumbs (Lemmers et al., 2004), Pals1/Stardust (Hurd et al., 2003), Par-3/Bazooka (Joberty et al., 2000), or EphrinB1 (Lee et al., 2008), would inhibit aPKC by restoring PS binding to the catalytic domain. Amazingly, the small increase in aPKC kinase activity from a Par-6-repressed state to a Cdc42-activated one is sufficient in promoting neuroblast polarity, revealing an amazing response of *Drosophila* neuroblasts to a small range in signal.

Cortical actin also plays a role in maintaining the localization and activation of aPKC in the neuroblast. Chapter five shows how Cdc42 regulates cortical actin. Cdc42 recruits Mbt to inactivate apical Tsr/cofilin and promote recruitment and activation of apical polarity and maintain separate apical and basal domains. *mbt* and *tsr* neuroblasts can still recruit polarity components to the neuroblast cortex but are unable to keep apical and basal domains separate. Interestingly, Mbt seems to function as an activator of aPKC activity as cortical aPKC in *mbt* neuroblasts is not sufficient to displace Miranda from the cortex. Tsr seems to have the opposite effect as *tsr* neuroblasts show cortical aPKC is active and can displace Miranda to the cytoplasm. Tsr/cofilin has been implicated in several tumor-related processes indicating an essential function of actin in maintaining distinct cortical domains in many cell types (Sidani et al., 2007; Wang et al., 2007b), and it is tantalizing to suspect an aPKC-depenent role for the generation of some of these tumors.

BIBLIOGRAPHY

Aceto, D., Beers, M. and Kemphues, K. J. (2006). Interaction of PAR-6 with CDC-42 is required for maintenance but not establishment of PAR asymmetry in C. elegans. *Dev Biol* **299**, 386-97.

Albertson, R. and Doe, C. Q. (2003). Dlg, Scrib and Lgl regulate neuroblast cell size and mitotic spindle asymmetry. *Nat Cell Biol* **5**, 166-70.

Andrianantoandro, E. and Pollard, T. D. (2006). Mechanism of actin filament turnover by severing and nucleation at different concentrations of ADF/cofilin. *Mol Cell* **24**, 13-23.

Artavanis-Tsakonas, S. and Simpson, P. (1991). Choosing a cell fate: a view from the Notch locus. *Trends Genet* **7**, 403-8.

Atwood, S. X., Chabu, C., Penkert, R. R., Doe, C. Q. and Prehoda, K. E. (2007). Cdc42 acts downstream of Bazooka to regulate neuroblast polarity through Par-6 aPKC. *J Cell Sci* **120**, 3200-6.

Barros, C. S., Phelps, C. B. and Brand, A. H. (2003). Drosophila nonmuscle myosin II promotes the asymmetric segregation of cell fate determinants by cortical exclusion rather than active transport. *Dev Cell* **5**, 829-40.

Beers, M. and Kemphues, K. (2006). Depletion of the co-chaperone CDC-37 reveals two modes of PAR-6 cortical association in C. elegans embryos. *Development* **133**, 3745-54.

Bellaiche, Y., Radovic, A., Woods, D. F., Hough, C. D., Parmentier, M. L., O'Kane, C. J., Bryant, P. J. and Schweisguth, F. (2001). The Partner of Inscuteable/Discs-large complex is required to establish planar polarity during asymmetric cell division in Drosophila. *Cell* **106**, 355-66.

Betschinger, J., Mechtler, K. and Knoblich, J. A. (2003). The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. *Nature* **422**, 326-30.

Betschinger, J., Mechtler, K. and Knoblich, J. A. (2006). Asymmetric segregation of the tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. *Cell* **124**, 1241-53.

Blair, A., Tomlinson, A., Pham, H., Gunsalus, K. C., Goldberg, M. L. and Laski, F. A. (2006). Twinstar, the Drosophila homolog of cofilin/ADF, is required for planar cell polarity patterning. *Development* **133**, 1789-97.

Broadus, J. and Doe, C. Q. (1997). Extrinsic cues, intrinsic cues and microfilaments regulate asymmetric protein localization in Drosophila neuroblasts. *Curr Biol* **7**, 827-35.

Buescher, M., Hing, F. S. and Chia, W. (2002). Formation of neuroblasts in the embryonic central nervous system of Drosophila melanogaster is controlled by SoxNeuro. *Development* **129**, 4193-203.

Cappello, S., Attardo, A., Wu, X., Iwasato, T., Itohara, S., Wilsch-Brauninger, M., Eilken, H. M., Rieger, M. A., Schroeder, T. T., Huttner, W. B. et al. (2006). The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface. *Nat Neurosci* **9**, 1099-107.

Chabu, C. and Doe, C. Q. (2008). Dap160/intersectin binds and activates aPKC to regulate cell polarity and cell cycle progression. *Development* **135**, 2739-46.

Chen, J., Godt, D., Gunsalus, K., Kiss, I., Goldberg, M. and Laski, F. A. (2001). Cofilin/ADF is required for cell motility during Drosophila ovary development and oogenesis. *Nat Cell Biol* **3**, 204-9.

Choksi, S. P., Southall, T. D., Bossing, T., Edoff, K., de Wit, E., Fischer, B. E., van Steensel, B., Micklem, G. and Brand, A. H. (2006). Prospero acts as a binary switch between self-renewal and differentiation in Drosophila neural stem cells. *Dev Cell* **11**, 775-89.

Diaz-Meco, M. T. and Moscat, J. (2001). MEK5, a new target of the atypical protein kinase C isoforms in mitogenic signaling. *Mol Cell Biol* **21**, 1218-27.

Doe, C. Q. (2008). Neural stem cells: balancing self-renewal with differentiation. *Development* **135**, 1575-87.

Dumstrei, K., Wang, F. and Hartenstein, V. (2003). Role of DE-cadherin in neuroblast proliferation, neural morphogenesis, and axon tract formation in Drosophila larval brain development. *J Neurosci* **23**, 3325-35.

Egger, B., Boone, J. Q., Stevens, N. R., Brand, A. H. and Doe, C. Q. (2007). Regulation of spindle orientation and neural stem cell fate in the Drosophila optic lobe. *Neural Develop* **2**, 1.

Egger, B., Chell, J. M. and Brand, A. H. (2008). Insights into neural stem cell biology from flies. *Philos Trans R Soc Lond B Biol Sci* **363**, 39-56.

Erben, V., Waldhuber, M., Langer, D., Fetka, I., Jansen, R. P. and Petritsch, C. (2008). Asymmetric localization of the adaptor protein Miranda in neuroblasts is achieved by diffusion and sequential interaction of Myosin II and VI. *J Cell Sci* **121**, 1403-14.

Etienne-Manneville, S. (2004). Cdc42--the centre of polarity. *J Cell Sci* **117**, 1291-300.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-35.

Fuerstenberg, S., Peng, C. Y., Alvarez-Ortiz, P., Hor, T. and Doe, C. Q. (1998). Identification of Miranda protein domains regulating asymmetric cortical localization, cargo binding, and cortical release. *Mol Cell Neurosci* **12**, 325-39.

Garrard, S. M., Capaldo, C. T., Gao, L., Rosen, M. K., Macara, I. G. and Tomchick, D. R. (2003). Structure of Cdc42 in a complex with the GTPase-binding domain of the cell polarity protein, Par6. *Embo J* **22**, 1125-33.

Garvalov, B. K., Flynn, K. C., Neukirchen, D., Meyn, L., Teusch, N., Wu, X., Brakebusch, C., Bamburg, J. R. and Bradke, F. (2007). Cdc42 regulates cofilin during the establishment of neuronal polarity. *J Neurosci* **27**, 13117-29.

Genova, J. L., Jong, S., Camp, J. T. and Fehon, R. G. (2000). Functional analysis of Cdc42 in actin filament assembly, epithelial morphogenesis, and cell signaling during Drosophila development. *Dev Biol* **221**, 181-94.

Gunsalus, K. C., Bonaccorsi, S., Williams, E., Verni, F., Gatti, M. and Goldberg, M. L. (1995). Mutations in twinstar, a Drosophila gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis. *J Cell Biol* **131**, 1243-59.

Harris, T. J. and Peifer, M. (2005). The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. *J Cell Biol* **170**, 813-23.

Hofmann, C., Shepelev, M. and Chernoff, J. (2004). The genetics of Pak. *J Cell Sci* **117**, 4343-54.

Hurd, T. W., Gao, L., Roh, M. H., Macara, I. G. and Margolis, B. (2003). Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. *Nat Cell Biol* **5**, 137-42.

Hutterer, A., Betschinger, J., Petronczki, M. and Knoblich, J. A. (2004). Sequential roles of Cdc42, Par-6, aPKC, and Lgl in the establishment of epithelial polarity during Drosophila embryogenesis. *Dev Cell* **6**, 845-54.

Ikeshima-Kataoka, H., Skeath, J. B., Nabeshima, Y., Doe, C. Q. and Matsuzaki, F. (1997). Miranda directs Prospero to a daughter cell during Drosophila asymmetric divisions. *Nature* **390**, 625-9.

Ito, K. and Hotta, Y. (1992). Proliferation pattern of postembryonic neuroblasts in the brain of Drosophila melanogaster. *Dev Biol* **149**, 134-48.

Izumi, Y., Ohta, N., Hisata, K., Raabe, T. and Matsuzaki, F. (2006). Drosophila Pins-binding protein Mud regulates spindle-polarity coupling and centrosome organization. *Nat Cell Biol* **8**, 586-93.

Joberty, G., Petersen, C., Gao, L. and Macara, I. G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat Cell Biol* **2**, 531-9.

Kay, A. J. and Hunter, C. P. (2001). CDC-42 regulates PAR protein localization and function to control cellular and embryonic polarity in C. elegans. *Curr Biol* **11**, 474-81.

Kheifets, V. and Mochly-Rosen, D. (2007). Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function. *Pharmacol Res* **55**, 467-76.

Knoblich, J. A. (2008). Mechanisms of asymmetric stem cell division. *Cell* **132**, 583-97.

Kojima, Y., Akimoto, K., Nagashima, Y., Ishiguro, H., Shirai, S., Chishima, T., Ichikawa, Y., Ishikawa, T., Sasaki, T., Kubota, Y. et al. (2008). The overexpression and altered localization of the atypical protein kinase C lambda/iota in breast cancer correlates with the pathologic type of these tumors. *Hum Pathol* **39**, 824-31.

Kosako, H., Yoshida, T., Matsumura, F., Ishizaki, T., Narumiya, S. and Inagaki, M. (2000). Rho-kinase/ROCK is involved in cytokinesis through the phosphorylation of myosin light chain and not ezrin/radixin/moesin proteins at the cleavage furrow. *Oncogene* **19**, 6059-64.

Kraut, R., Chia, W., Jan, L. Y., Jan, Y. N. and Knoblich, J. A. (1996). Role of inscuteable in orienting asymmetric cell divisions in Drosophila. *Nature* **383**, 50-5.

Langevin, J., Le Borgne, R., Rosenfeld, F., Gho, M., Schweisguth, F. and Bellaiche, Y. (2005). Lethal giant larvae controls the localization of notch-signaling regulators numb, neuralized, and Sanpodo in Drosophila sensory-organ precursor cells. *Curr Biol* **15**, 955-62.

Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. and Parker, P. J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281**, 2042-5.

Lee, C. Y., Andersen, R. O., Cabernard, C., Manning, L., Tran, K. D., Lanskey, M. J., Bashirullah, A. and Doe, C. Q. (2006a). Drosophila Aurora-A kinase inhibits neuroblast self-renewal by regulating aPKC/Numb cortical polarity and spindle orientation. *Genes Dev* **20**, 3464-74.

Lee, C. Y., Robinson, K. J. and Doe, C. Q. (2006b). Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. *Nature* **439**, 594-8.

Lee, C. Y., Wilkinson, B. D., Siegrist, S. E., Wharton, R. P. and Doe, C. Q. (2006c). Brat is a Miranda cargo protein that promotes neuronal differentiation and inhibits neuroblast self-renewal. *Dev Cell* **10**, 441-9.

Lee, H. S., Nishanian, T. G., Mood, K., Bong, Y. S. and Daar, I. O. (2008). EphrinB1 controls cell-cell junctions through the Par polarity complex. *Nat Cell Biol.*

Lemmers, C., Michel, D., Lane-Guermonprez, L., Delgrossi, M. H., Medina, E., Arsanto, J. P. and Le Bivic, A. (2004). CRB3 binds directly to Par6 and regulates the morphogenesis of the tight junctions in mammalian epithelial cells. *Mol Biol Cell* **15**, 1324-33.

Li, Q., Wang, J. M., Liu, C., Xiao, B. L., Lu, J. X. and Zou, S. Q. (2008). Correlation of aPKC-iota and E-cadherin expression with invasion and prognosis of cholangiocarcinoma. *Hepatobiliary Pancreat Dis Int* **7**, 70-5.

Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D. and Pawson, T. (2000). A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat Cell Biol* **2**, 540-7.

Liu, S. L., Fewkes, N., Ricketson, D., Penkert, R. R. and Prehoda, K. E. (2008). Filament-dependent and -independent localization modes of Drosophila non-muscle myosin II. *J Biol Chem* **283**, 380-7.

Lu, B., Rothenberg, M., Jan, L. Y. and Jan, Y. N. (1998). Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in Drosophila neural and muscle progenitors. *Cell* **95**, 225-35.

Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev* **8**, 1787-802.

Matsumura, F. (2005). Regulation of myosin II during cytokinesis in higher eukaryotes. *Trends Cell Biol* **15**, 371-7.

Matsuzaki, F., Ohshiro, T., Ikeshima-Kataoka, H. and Izumi, H. (1998). miranda localizes staufen and prospero asymmetrically in mitotic neuroblasts and epithelial cells in early Drosophila embryogenesis. *Development* **125**, 4089-98.

Melzig, J., Rein, K. H., Schafer, U., Pfister, H., Jackle, H., Heisenberg, M. and Raabe, T. (1998). A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the Drosophila adult central nervous system. *Curr Biol* **8**, 1223-6.

Menzel, N., Schneeberger, D. and Raabe, T. (2007). The Drosophila p21 activated kinase Mbt regulates the actin cytoskeleton and adherens junctions to control photoreceptor cell morphogenesis. *Mech Dev* **124**, 78-90.

Messerschmidt, A., Macieira, S., Velarde, M., Badeker, M., Benda, C., Jestel, A., Brandstetter, H., Neuefeind, T. and Blaesse, M. (2005). Crystal structure of the catalytic domain of human atypical protein kinase C-iota reveals interaction mode of phosphorylation site in turn motif. *J Mol Biol* **352**, 918-31.

Moriyama, K., Iida, K. and Yahara, I. (1996). Phosphorylation of Ser-3 of cofilin regulates its essential function on actin. *Genes Cells* **1**, 73-86.

Moscat, J. and Diaz-Meco, M. T. (2000). The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. *EMBO Rep* **1**, 399-403.

Nagai-Tamai, Y., Mizuno, K., Hirose, T., Suzuki, A. and Ohno, S. (2002). Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells. *Genes Cells* **7**, 1161-71.

Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993). Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **268**, 13-6.

Nipper, R. W., Siller, K. H., Smith, N. R., Doe, C. Q. and Prehoda, K. E. (2007). Galphai generates multiple Pins activation states to link cortical polarity and spindle orientation in Drosophila neuroblasts. *Proc Natl Acad Sci U S A* **104**, 14306-11.

Noda, Y., Kohjima, M., Izaki, T., Ota, K., Yoshinaga, S., Inagaki, F., Ito, T. and Sumimoto, H. (2003). Molecular recognition in dimerization between PB1 domains. *J Biol Chem* **278**, 43516-24.

Ohshiro, T., Yagami, T., Zhang, C. and Matsuzaki, F. (2000). Role of cortical tumour-suppressor proteins in asymmetric division of Drosophila neuroblast. *Nature* **408**, 593-6.

Overton, P. M., Meadows, L. A., Urban, J. and Russell, S. (2002). Evidence for differential and redundant function of the Sox genes Dichaete and SoxN during CNS development in Drosophila. *Development* **129**, 4219-28.

Park, Y., Rangel, C., Reynolds, M. M., Caldwell, M. C., Johns, M., Nayak, M., Welsh, C. J., McDermott, S. and Datta, S. (2003). Drosophila perlecan modulates FGF and hedgehog signals to activate neural stem cell division. *Dev Biol* **253**, 247-57.

Pears, C., Schaap, D. and Parker, P. J. (1991). The regulatory domain of protein kinase C-epsilon restricts the catalytic-domain-specificity. *Biochem J* **276 (Pt 1)**, 257-60.

Pearson, B. J. and Doe, C. Q. (2003). Regulation of neuroblast competence in Drosophila. *Nature* **425**, 624-8.

Peng, C. Y., Manning, L., Albertson, R. and Doe, C. Q. (2000). The tumour-suppressor genes lgl and dlg regulate basal protein targeting in Drosophila neuroblasts. *Nature* **408**, 596-600.

Penkert, R. R., DiVittorio, H. M. and Prehoda, K. E. (2004). Internal recognition through PDZ domain plasticity in the Par-6-Pals1 complex. *Nat Struct Mol Biol* **11**, 1122-7.

Peterson, F. C., Penkert, R. R., Volkman, B. F. and Prehoda, K. E. (2004). Cdc42 regulates the Par-6 PDZ domain through an allosteric CRIB-PDZ transition. *Mol Cell* **13**, 665-76.

Petronczki, M. and Knoblich, J. A. (2001). DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in Drosophila. *Nat Cell Biol* **3**, 43-9.

Qian, Y. and Prehoda, K. E. (2006). Interdomain interactions in the tumor suppressor discs large regulate binding to the synaptic protein GukHolder. *J Biol Chem* **281**, 35757-63.

Regala, R. P., Weems, C., Jamieson, L., Khoor, A., Edell, E. S., Lohse, C. M. and Fields, A. P. (2005). Atypical protein kinase C iota is an oncogene in human non-small cell lung cancer. *Cancer Res* **65**, 8905-11.

Rolls, M. M., Albertson, R., Shih, H. P., Lee, C. Y. and Doe, C. Q. (2003). Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. *J Cell Biol* **163**, 1089-98.

Schaefer, M., Shevchenko, A., Shevchenko, A. and Knoblich, J. A. (2000). A protein complex containing Inscuteable and the Galpha-binding protein Pins orients asymmetric cell divisions in Drosophila. *Curr Biol* **10**, 353-62.

Schneeberger, D. and Raabe, T. (2003). Mbt, a Drosophila PAK protein, combines with Cdc42 to regulate photoreceptor cell morphogenesis. *Development* **130**, 427-37.

Schober, M., Schaefer, M. and Knoblich, J. A. (1999). Bazooka recruits Inscuteable to orient asymmetric cell divisions in Drosophila neuroblasts. *Nature* **402**, 548-51.

Schonegg, S. and Hyman, A. A. (2006). CDC-42 and RHO-1 coordinate acto-myosin contractility and PAR protein localization during polarity establishment in C. elegans embryos. *Development* **133**, 3507-16.

Shen, C. P., Jan, L. Y. and Jan, Y. N. (1997). Miranda is required for the asymmetric localization of Prospero during mitosis in Drosophila. *Cell* **90**, 449-58.

Shen, C. P., Knoblich, J. A., Chan, Y. M., Jiang, M. M., Jan, L. Y. and Jan, Y. N. (1998). Miranda as a multidomain adapter linking apically localized Inscuteable and basally localized Staufen and Prospero during asymmetric cell division in Drosophila. *Genes Dev* **12**, 1837-46.

Sidani, M., Wessels, D., Mouneimne, G., Ghosh, M., Goswami, S., Sarmiento, C., Wang, W., Kuhl, S., El-Sibai, M., Backer, J. M. et al. (2007). Cofilin determines the migration behavior and turning frequency of metastatic cancer cells. *J Cell Biol* **179**, 777-91.

Siegrist, S. E. and Doe, C. Q. (2005). Microtubule-induced Pins/Galphai cortical polarity in Drosophila neuroblasts. *Cell* **123**, 1323-35.

Siegrist, S. E. and Doe, C. Q. (2006). Extrinsic cues orient the cell division axis in Drosophila embryonic neuroblasts. *Development* **133**, 529-36.

Siller, K. H., Cabernard, C. and Doe, C. Q. (2006). The NuMA-related Mud protein binds Pins and regulates spindle orientation in Drosophila neuroblasts. *Nat Cell Biol* **8**, 594-600.

Smith, C. A., Lau, K. M., Rahmani, Z., Dho, S. E., Brothers, G., She, Y. M., Berry, D. M., Bonneil, E., Thibault, P., Schweisguth, F. et al. (2007). aPKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. *Embo J* **26**, 468-80.

Spitaler, M., Villunger, A., Grunicke, H. and Uberall, F. (2000). Unique structural and functional properties of the ATP-binding domain of atypical protein kinase C-iota. *J Biol Chem* **275**, 33289-96.

Standaert, M. L., Bandyopadhyay, G., Kanoh, Y., Sajan, M. P. and Farese, R. V. (2001). Insulin and PIP3 activate PKC-zeta by mechanisms that are both dependent and independent of phosphorylation of activation loop (T410) and autophosphorylation (T560) sites. *Biochemistry* **40**, 249-55.

Suzuki, A., Akimoto, K. and Ohno, S. (2003). Protein kinase C lambda/iota (PKClambda/iota): a PKC isotype essential for the development of multicellular organisms. *J Biochem* **133**, 9-16.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-72.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-76.

Tian, A. G. and Deng, W. M. (2008). Lgl and its phosphorylation by aPKC regulate oocyte polarity formation in Drosophila. *Development* **135**, 463-71.

Wang, H., Ouyang, Y., Somers, W. G., Chia, W. and Lu, B. (2007a). Polo inhibits progenitor self-renewal and regulates Numb asymmetry by phosphorylating Pon. *Nature* **449**, 96-100.

Wang, H., Somers, G. W., Bashirullah, A., Heberlein, U., Yu, F. and Chia, W. (2006). Aurora-A acts as a tumor suppressor and regulates self-renewal of Drosophila neuroblasts. *Genes Dev* **20**, 3453-63.

Wang, W., Eddy, R. and Condeelis, J. (2007b). The cofilin pathway in breast cancer invasion and metastasis. *Nat Rev Cancer* **7**, 429-40.

Weiner, O. D., Neilsen, P. O., Prestwich, G. D., Kirschner, M. W., Cantley, L. C. and Bourne, H. R. (2002). A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. *Nat Cell Biol* **4**, 509-13.

Wodarz, A., Ramrath, A., Grimm, A. and Knust, E. (2000). Drosophila atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J Cell Biol* **150**, 1361-74.

Wodarz, A., Ramrath, A., Kuchinke, U. and Knust, E. (1999). Bazooka provides an apical cue for Inscuteable localization in Drosophila neuroblasts. *Nature* **402**, 544-7.

Yamanaka, T., Horikoshi, Y., Suzuki, A., Sugiyama, Y., Kitamura, K., Maniwa, R., Nagai, Y., Yamashita, A., Hirose, T., Ishikawa, H. et al. (2001). PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. *Genes Cells* **6**, 721-31.

Yoon, K. and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat Neurosci* **8**, 709-15.

Yu, F., Kuo, C. T. and Jan, Y. N. (2006). Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology. *Neuron* **51**, 13-20.

Yu, F., Morin, X., Cai, Y., Yang, X. and Chia, W. (2000). Analysis of partner of inscuteable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in inscuteable apical localization. *Cell* **100**, 399-409.

Zhang, H. and Macara, I. G. (2006). The polarity protein PAR-3 and TIAM1 cooperate in dendritic spine morphogenesis. *Nat Cell Biol* **8**, 227-37.

Zhang, X., Zhu, J., Yang, G. Y., Wang, Q. J., Qian, L., Chen, Y. M., Chen, F., Tao, Y., Hu, H. S., Wang, T. et al. (2007). Dishevelled promotes axon differentiation by regulating atypical protein kinase C. *Nat Cell Biol* **9**, 743-54.

Zhao, Z. S. and Manser, E. (2005). PAK and other Rho-associated kinases--effectors with surprisingly diverse mechanisms of regulation. *Biochem J* **386**, 201-14.

Zhu, C. C., Boone, J. Q., Jensen, P. A., Hanna, S., Podemski, L., Locke, J., Doe, C. Q. and O'Connor, M. B. (2008). Drosophila Activin- and the Activin-like product Dawdle function redundantly to regulate proliferation in the larval brain. *Development* **135**, 513-21.