# PROTEIN-LIPID AND PROTEIN-PROTEIN INTERACTIONS COOPERATE TO POLARIZE THE PAR COMPLEX IN DROSOPHILA NEUROBLASTS

by

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DISSERTATION ABSTRACT

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Title: Protein-Lipid and Protein-Protein Interactions Cooperate to Polarize the Par Complex in Drosophila Neuroblasts

Animal cells display a variety of shapes and functions that require specific cellular machinery to achieve. For many cells, functions are derived by the molecularly distinct collections of molecules in different regions. For stem cells, partitioning of membrane components is key to producing daughter cells with distinct fates. The evolutionarily conserved Par complex consisting of the kinase aPKC and its adaptor protein Par6 through regulation of the PDZ scaffold Par3 and the small GTPase Cdc42 localizes its activity to the apical cortex of these cells, setting up mutually exclusive domains by inhibiting localization of basal determinants to the Par domain through aPKC kinase activity.

In this work, I have set out to determine the specific, direct interactions that are required to polarize the Par complex. Previously, it had been shown that Par3 and/or Cdc42 were required and sufficient for cortical targeting and polarization of the Par complex in a variety of cell types and organisms. There were also multiple reported interactions between the Par complex and Par3; however the requirement and contribution of each had not been determined.

Through this work, I have shown that there is an additional interaction that aPKC must have for cortical targeting and polarization, the C1 domain of aPKC with cortical phospholipids. Additionally as this domain has the ability to bind constitutively to the cortex, it must be regulated intramolecularly through the PB1 and kinase domains of aPKC.

I have also shown that the previously unidentified Par3 PDZ2:aPKC PBM interaction is required for aPKC polarization in *Drosophila* neuroblasts, as mutation of the PBM leads to cytoplasmic, inactive aPKC. This suggests that the previously reported reactions are not required, at least in neuroblasts for polarization.

Together these discoveries help us to better understand the minimal interactions that are required for polarization of the Par complex.

This dissertation includes both previously published/unpublished and co-authored material.

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# REGULATION OF PAR COMPLEX LOCALIZATION

This chapter contains unpublished material written by K.A.J.

Chapter II contains unpublished, co-authored material.

Chapter III contains published, co-authored material.

## INTRODUCTION

During development, how do complex, multicellular organisms with an intricate network of different cells types, organs, and processes emerge from a single cell zygote? How do they maintain their tissues and cellular functions during homeostasis? A key part of these questions is: how do cells make decisions based on their environments to choose what fates or functions they will have. For many cells, the answer is through cell polarity.

Cell polarity specifically refers to the asymmetric distribution of cellular components to allow for regionalization of functions within a cell or for differences of cell fate when undergoing mitosis. This can include proteins, RNAs, organelles, cytoskeletal components, and even lipids within the plasma membrane (Nelson, 2003).

## ANIMAL CELLS POLARIZE TO PERFORM SPECIFIC TASKS OR TO YIELD DIFFERENT FATES

Many of the cells within our bodies and other multicellular animals display cell polarity to do their specific jobs or to lend specific fates to dividing cells. Neurons

display polarity in their shape (they have a cell body with many small dendrites that receive and process input signals and a single long axon) and in their function (signals move in a unidirectional manner down this axon away from the cell body to deliver messages to another neuron). Migrating cells have a leading edge that interacts with and samples the environment looking for cues both attractive and repulsive to determine the proper direction to travel. Epithelia or sheets of cells line all of the regions of the body that are exposed to the environment: skin, organs such as intestines, etc. These cells exhibit functional polarity where the apical region that is exposed to the environment has roles such as absorption or secretion, while the lateral sides of the cell are involved in providing barrier function through the formation of junctions, and the basal sides are involved with interacting with the basement membrane and underlying connective tissue. Additionally stem cells display polarity in their divisions where the two daughter cells must be given different fate determinants, such that one cell will remain a stem cell and the other cell will have a more differentiated fate (Nelson, 2003).

What are the proteins that set up cell polarity? Are they different across different cellular contexts? Or is there a commonality to them? Work over the past few decades is helping to shed light on the molecules and molecular mechanisms underlying cell polarity in animals.

## ANIMAL CELLS RELY ON THE EVOLUTIONARILY CONSERVED PAR COMPLEX

### The Par complex localizes asymmetrically in polarized cells

Organisms across the animal kingdom use the evolutionarily conserved Par complex for many of these polarized cellular processes. The Par complex consists of the kinase aPKC (atypical Protein Kinase C) and its binding partner, the PDZ-domain containing scaffold Par6. This complex is the core component of cell polarity across different organisms and cell types. Indeed it is the activity of aPKC that is the main driver of cell polarity (Heinrique and Schweisguth, 2003). Therefore understanding how the Par complex is localized and more specifically how aPKC is regulated is key to understanding how cells polarize.

In all polarized cell types, the Par complex localizes asymmetrically to one side of a cell excluding downstream determinants to an opposing domain. In the above examples, the Par complex would localize to the growth cone of the developing axon of a neuron as it moves towards its synaptic neuronal partner, along the leading edge of a migratory cell regulating the underlying cytoskeleton to move the cell in the right direction, along the apical side of epithelia where it specifies components for exchange with the environment, in the apical domain of stem cells, such as *Drosophila* neuroblasts, where it excludes fate determinants to the basal domain allowing for the creation of another stem cell and a differentiated cell upon division, and in the anterior region of *C. elegans* one cell embryos, which sets up the future body plan and cell fates of the developing organism (Campanale et al., 2017; Goldstein and Macara, 2007).

**aPKC kinase activity creates mutually exclusive polarity domains**

Once the Par complex localizes to one region of a cell, how does is it able to set up opposing cortical domains? The main output of the Par complex is aPKC activity.

Although it was known that aPKC could phosphorylate its substrates, the mechanism of how this caused them to be excluded from the Par domain was not determined until recently. Many aPKC substrates contain their aPKC phosphorylation sites within regions that have high basic-hydrophobic character. These regions have been shown to be able to bind to cortical phospholipids. However upon phosphorylation, net negative charge is introduced in these regions causing electrostatic repulsion from the membrane and releasing them from the Par domain (Bailey and Prehoda, 2015; Dong et al., 2015). How these substrates are then localized to the opposing membrane is not fully understood may involve phosphatases that remove the phosphorylations (Sousa-Nunes et al., 2009; Zhang et al., 2016).

It is very important to regulate this aPKC kinase activity as it has been shown that mislocalization of activity can have drastic effects, including gross morphology and tissue overgrowth defects (Reina-Campos et al., 2019; Wirtz-Peitz et al., 2008). Many different regulatory pathways and complexes that feed into Par polarization have been discovered with many also being conserved across cell types, such as the basolateral Scrib complex, the apical Crumbs and Stardust complex. However these complexes tend to regulate the maintenance of Par polarity or set the size of the domains (Campanale et al., 2017). Here I will focus on the components required for establishing polarity.

The main regulators of Par polarity establishment across cells is the PDZ-domain containing scaffold Par3 and the small GTPase Cdc42. Although the Par complex also interacts with a variety of other regulatory factors and complexes, these two proteins appear over and over as key upstream components in Par complex regulation (Goldstein and Macara, 2007).

## PAR COMPLEX HISTORY

The Par or *partitioning defective* proteins were first discovered in a screen looking for partitioning defects of germ particles (P-granules) in the *C. elegans* one cell stage embryos. Localization of these P-granules to the posterior of the cell is known to be a key feature of the first cell division, as the posterior daughter cell (or P2) will go on to define the future germ line (Kemphues et al., 1988, Kemphues, 2000). Par3 was one of the first of these Par genes to have its localization be described. It was shown to localize to the anterior cortex and worked in a negative feedback loop with posterior Par proteins such as Par1 and Par2. Additionally it appeared that Par3 was able to localize onto the cortex independent of the other four Par proteins (Etemad-Moghadam et al., 1995). An additional protein, Par6 was discovered in 1996 and showed a similar ability to restrict posterior Par proteins from the anterior cortex. Unlike the other Par proteins discovered to date, it appeared to be partially required for Par3 localization, as Par-3 was shown to be weakly punctate on the cortex during the early one-cell stage but cytoplasmic in the late one-cell stage in a *par-6* mutant embryo (Watts et al., 1996).

The first of these proteins to be found outside of *C. elegans* was the Par3 homologue Bazooka. The Bazooka gene had originally been discovered in a screen for lethal cuticle defects in *Drosophila* embryogenesis and was later shown to be required for early zonula adherens (ZA, *Drosophila* tight junctions) formation and polarity of the blastoderm epithelia (Müller et al., 1996; Wieschaus et al., 1984). When it was finally cloned, it was discovered that it shared strong sequence similarity with *C. elegans* Par3 and was shown to localize to the apical membrane of embryonic epithelia and neuroblasts in *Drosophila* (Kuchinke et al., 1998). The finding of a mammalian homologue of Par3

(originally named ASIP) through its interaction with aPKC led to the discovery that aPKC itself colocalized with Par3 near the junctions of MDCK cells and directly interacted with Par3 (Izumi et al., 1998). Later this same year, the same group published another report describing the *C. elegans* aPKC (PKC-3), its direct interaction with Par3, and its anterior localization (Tabuse et al., 1998). The conservation of Par3 and aPKC and their coordinated roles in polarity in *C. elegans* and mammals led to the identification of aPKC in *Drosophila* where it again showed the ability to interact with Par3 and polarize to the apical domains of epithelia and neuroblasts (Wodarz et al., 2000).

The discovery that aPKC was involved in polarity was the first *in vivo* role that was determined for it although the first atypical PKC was cloned and described in the late 1980's (Ono et al., 1988; Ono et al., 1989; Tabuse et al., 1998). aPKC is unique compared to other PKCs. Conventional and novel PKCs all contain at least one C1 domain that is activated by binding to the lipid diacylglycerol (DAG) or phorbol esters and a C2 domain that is activated by $Ca^{2+}$ binding. Activation of these domains allows for lipid interactions and maturation of the kinases (Rosse et al., 2010). Although aPKC has a C1 domain, it is considered atypical as it is not activated by DAG due to charged interactions located along the cleft that would normally bind to it (Pu et al., 2006). aPKCs also have an N-terminal PB1 domain that allows for binding to other proteins that contain PB1 domains (Noda et al., 2003).

The questions of how aPKC was regulated without the known $Ca^{2+}$ and DAG binding seen in other conventional and novel PKCs and how it exerted its relevant function in cells were not well understood (Shieh et al, 2002).

A partial answer came with the discovery of the PDZ protein Par6 in *C. elegans*. This protein was shown to be a binding partner of aPKC that was conserved in *Drosophila* and mammals (Hung et al., 1999). Further analysis in mammals led to the discovery of Cdc42 as a direct binding partner of Par6 and thus a regulator of Par polarity (Joberty et al., 2000; Lin et al., 2000). The conservation of Cdc42 as a Par complex regulator was quickly confirmed in *Drosophila* and *C. elegans* (Hutterer et al., 2004; Kay and Hunter, 2001; Petronczki and Knoblich, 2001).

The small GTPase Cdc42 had been known to be required for polarity of bud site formation in budding yeast *Saccharomyces cerevisiae* (Adams et al., 1990). This protein serves as a key determiner of signals, taking in inputs from a variety of signals throughout different cell types and organisms, and leading to different downstream effectors in polarity cascades (Etienne-Manneville and Hall, 2002; Etienne-Manneville, 2004).

Cdc42 itself has been shown to be highly regulated. As a GTPase, it cycles between inactive and active states based upon the state of the nucleotide that it binds. Cdc42 with GTP is active and regulation by a GAP (GTPase-activating protein) causes hydrolysis of the GTP to GDP, rendering CDC42 inactive. Activation occurs through interacting with a GEF (guanine nucleotide exchange factor) that causes Cdc42 to exchange the GDP for a GTP molecule (Etienne-Manneville and Hall, 2002). Yeast studies have shown that this cycling activity is key for Cdc42's role in polarization of yeast cells and regulation of the GEFs and GAPs for Cdc42 are key to this ability (Irazoqui et al., 2003).

## PAR COMPLEX INTERACTIONS AND ACTIVITY REGULATION

We know that aPKC/Par6, Par3, and Cdc42 all localize in the same regions of polarized cells and have similar phenotypes when disrupted. Early research also suggested that there were interdependent requirements between them for their localization (Aceto et al., 2006; Etemad-Moghadam et al., 1995; Hung et al., 1999; Tabuse et al., 1998; Watts et al., 1996). But how do these individual components interact with one another?

aPKC and Par6 share a direct interaction between their N-terminal regions. This was originally mapped using yeast-two hybrid and immunoprecipitation from lysates (Joberty et al, 2000; Suzuki et al, 2001). It was later confirmed that this interaction relied on the heterodimerization of the PB1 domains within each protein (Hirano et al, 2005; Noda et al., 2003). These proteins are interdependent in their localization in all Par polarized settings to date (Lang and Munro, 2017). Additionally it has been shown that Par6 may be unstable when not in complex with aPKC (Nunes de Almeida et al, 2019).

A huge discovery came when Par6 was found to be a direct effector of Cdc42. This connected the cell polarity functions of Cdc42 known from yeast to Par-mediated polarity. Through screens for Cdc42 effectors, it was discovered that Par6 is a direct interactor of active Cdc42 and binds using its semi-CRIB and PDZ domains (Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2001).

A key finding in understanding how Cdc42 contributed to Par complex regulation came from a crystal structure of constitutively active Cdc42 (Q61L) in complex with Par6. This structure showed that Cdc42 caused a conformational change in Par6 upon binding and required both the semi-CRIB and the first part of the PDZ domain to

facilitate binding (Garrard et al., 2003). As the Par complex was thought to be inhibited when alone, a model emerged where binding to active Cdc42 through Par6 allowed for activation of aPKC activity (Yamanaka et al., 2001). The Cdc42:Par6 structure also showed that the PDZ domain was available for binding to other proteins (Garrard et al., 2003). Further work connected this conformational change to the Par6 PDZ Binding Motif (PBM) binding ability. It was shown that the PDZ domain of Par6 did not fully match the conformational structure of other known PDZ domains but upon binding to Cdc42, a conformational switch forced it to adopt a conventional PDZ structure and also increased its affinity for PBM ligands (Peterson et al., 2004; Whitney et al., 2011). Thus Cdc42 could serve to both localize and activate the Par complex with further refinement of this localization possible through the PDZ domain of Par6 (Heinrique and Schweisguth, 2003; Pichaud et al., 2019).

Par3 has been found to interact with both aPKC and Par6 through multiple interactions. The first of these interactions was discovered to be between aPKC's kinase domain and a conserved region of the C-terminal tail region (CR3) of Par3 (Izumi et al., 1998). Around this same time, another interaction was shown to occur between aPKC and the PDZ2-PDZ3 region of Par3; however this was not mapped any further (Wodarz et al., 2000).

It was later discovered that the CR3 region of Par3 contains aPKC phosphorylation sites that regulates the interaction with aPKC itself and is required for the formation of junctions in epithelia (Lin et al., 2000; Morais-de-Sá et al., 2010; Nagai-Tamai, et al., 2002). However it was also shown that addition of Par3's aPKC interaction region led to a decrease in aPKC activity for other substrates, which led the authors to

conclude that Par3 can also act as an inhibitor of aPKC activity (Lin et al., 2000). This led to a paradigm in the field of how Par3 could be a substrate and be an inhibitor and led to speculation that Par3 inhibition upon phosphorylation could help to refine aPKC activity spatiotemporally (Heinrique and Schweisguth, 2003). Recently another group returned to this idea of inhibition when they were unable to phosphorylate the CR3 of Par3 (but were able to phosphorylate another aPKC substrate) and were able to obtain a stable crystal structure of this region in complex with aPKC kinase domain. They proposed that Par3 forms an inactive complex with aPKC that requires a secondary activation for aPKC to phosphorylate Par3. This speculated that this would allow Par3 to localize the Par complex to the apical domain before it was itself phosphorylated and moved to the junctions (Soriano et al., 2016). However this model was refuted by a higher sensitivity assay that concluded that Par3 is a substrate and competes with other substrates for phosphorylation, which explained the previously seen inhibitory effects (Holly and Prehoda, 2019).

An interaction between Par6 and Par3 was shown to require the PDZ domain of Par6 and the PDZ1 domain of Par3 (Joberty et al., 2000; Lin et al., 2000). However a later study showed that PDZ1 was dispensable for localization of Par3 in *C.elegans* but instead PDZ2 was required for development (Li et al., 2010a). The most recent addition to this menagerie of Par3/Par complex interactions is between a previously unidentified PDZ PBM to the PDZ1 and PDZ3 domains of Par3. The authors sought to determine if Par3 and Par6 did in fact interact, as the previous idea of PDZ stacking interactions between these two proteins was debated. They showed that Par6 contains a previously unidentified PDZ PBM that is able to interact with either PDZ1 or PDZ3 of Par3, and that

*in vitro*, Par3 could interact with two of these PBMs at the same time (Renschler et al., 2018). This could allow for the formation of higher order polarity complexes as has been proposed to occur *in vivo* (Dickinson et al., 2017).

## REGULATION OF PAR COMPLEX LOCALIZATION

### How does the Par complex get to the membrane?

The key step in Par-mediated polarity is getting aPKC to a specific area of the cortex and ensuring that it is only active there. This process relies on a carefully regulated system of control. Although the Par complex can interact with a variety of polarity regulatory complexes and other proteins, experimental evidence across multiple organisms has shown that the Par complex relies chiefly on Par3 and Cdc42 for its localization (as well as its activity) (Lang and Munro, 2017). Other inputs seem to hone the signal for specific contexts or maintenance of the complex or in cases like the *C. elegans* one cell embryo define the boundary of the Par domain through mutual exclusion from an opposing domain (Goldstein and Macara, 2007).

Initial localization of the Par complex appears to depend on Par3 and/or Cdc42. Although whether both regulators are required, which order they are required in, and if these proteins also regulate each other appear to be context specific and are still matters of debate (Lang and Munro, 2017).

### Par complex regulation in different cellular contexts

To understand whether Par complex recruitment and regulation have a common mechanism across different models, I will review what is known regarding the

requirement for Par3 and Cdc42 across a variety of contexts, focusing mostly on data from the *C. elegans* one cell zygote and different tissues in *Drosophila*, as most of what is known about polarity regulation has been studied in these systems. I will also touch on what is known in mammalian epithelia. Additionally I will discuss what is known about the regulation of Par3 and Cdc42 themselves in these contexts and whether there is any feedback between them.

### *C. elegans zygote*

As mentioned above, the Par complex was first discovered in the *C. elegans* zygote with homologues quickly discovered across the metazoan (Goldstein and Macara, 2007). Thus it is no surprise that this is the best studied context for understanding Par complex polarity and its regulation.

In *C. elegans* zygotes, polarization has two phases: establishment and maintenance (Cuenca et al., 2003). Before fertilization, the Par complex, Par3 and Cdc42 localize to the entire cortex. Why these proteins start out cortical is not well understood but is beginning to be teased apart (Reich et al., 2019). It is not until fertilization occurs and local relaxation of the actomyosin cytoskeleton occurs in the posterior region that anterior polarization of the Par complex occurs (Cowan and Hyman, 2007; Munro et al., 2004). This is referred to as the establishment phase.

During establishment both Par3 and Cdc42 are believed to be required for localizing the Par complex onto the membrane. This is thought to occur through the creation of two distinct pools: one with Par3 clusters that has inactive Par complex and one with active more diffuse clusters with Cdc42 that have active Par complex (Lang and

Munro, 2017; Rodriguez et al., 2017; Wang et al., 2017). It is thought that Par3 helps to recruit the Par complex to Cdc42 on the cortex through a cycling mechanism that switches the Par complex between them (Rodriguez et al., 2017). Exactly how this cycling occurs is not fully understood. It is thought that the clustering of Par3 with the Par complex allows it to polarize by being pulled along with the cortical flows (Rodriguez et al., 2017).

Although these cortical flows are initial started by the process of fertilization, Cdc42 is able to maintain them through a positive feedback loop in maintenance stage of polarity (Kumfer et al., 2010). It is also during the maintenance stage that Par3 puncta become smaller and a gradient of Cdc42:Par complex is maintained through mutual interactions with posterior polarity proteins (Lang and Munro, 2017; Rodriguez et al., 2017; Wang et al., 2017).

How Par3 and Cdc42 themselves get to the membrane is not fully understood. Par3 is known to be able to oligomerize through its N-terminal CR1 domain, and this is thought to be important for its membrane localization (Feng et al., 2007). Although it is also known to be able to bind directly to lipids (Yu and Harris, 2012).

The regulation of Cdc42 membrane localization is not known but is not reliant on Par3 or the Par complex (Lang and Munro, 2017).

### *Drosophila neuroblasts*

Besides the *C. elegans* one cell zygote, *Drosophila* neuroblasts are perhaps the next best studied system for understanding Par-mediated polarity and its regulation. In *Drosophila* neuroblasts, cell polarity is tightly coupled to the cell cycle and spindle

orientation. This allows for the division plane to separate the opposing cortical domains. These cells also undergo asymmetric cell division, where the larger daughter cell inherits the apical, Par domain and the smaller daughter cell inherits the basal fate determinants (Prehoda, 2009).

The Par complex is initially cytoplasmic in interphase, but upon entry into mitosis, it is quickly recruited and activated within the apical domain (Oon and Prehoda, 2019). In these cells, Cdc42 has been shown to remain on the cortex throughout the cell cycle, enriching in the apical domain during metaphase. Although it has not been shown, it is thought that Cdc42 is only active in the apical domain. Par3 is cytoplasmic and punctate in a depolarized manner in interphase. It is then recruited to the apical domain upon entry into mitosis, but it appears that it may be recruited before the Par complex, as Par3 mutant metaphase neuroblasts have cytoplasmic Par complex (Atwood et al., 2007; Oon and Prehoda, 2019).

Cdc42 and Par3 appear to be required non-redundantly for Par complex polarization, as mutants of either protein show cytoplasmic aPKC and Par6. Additionally it appears that Cdc42 has no role in recruiting Par3, although there is some evidence that the reverse is not true and Par3 may have a role in Cdc42 regulation (Atwood et al, 2007).

***Drosophila photoreceptors***

In *Drosophila* photoreceptors, development relies on establishment of apical identity which allows for the creation of zonula adherens (ZA, tight junctions of *Drosophila*) and proper morphogenesis (Pichaud, 2018). In these cells, Par3 localizes to

these ZA while Cdc42 localizes to the apical domain. In addition to Cdc42 and Par3, the apical transmembrane protein Crumbs (Crb) is often implicated in photoreceptor and epithelial polarity regulation (Nunes de Almeida, 2019).

It has been recently shown that localization of the Par complex to the apical domain relies on Cdc42. Cdc42's apical localization is independent of the Par complex and Par3 by an unknown mechanism. Interestingly Par3 localization to the ZA relies on Cdc42, although the mechanism behind this regulation and whether it is direct has not been determined. Although the Par complex cannot localize to the apical domain in the absence of Cdc42, it can still get to the membrane through interacting with Par3 at the ZA (Nunes de Almeida et al., 2019). This Cdc42 independent interaction of the Par complex with Par3 could be due through the PBM of Par6 (Renschler et al, 2018). Additionally it was shown that Crb was not required for cortical targeting of the Par complex but does serve to stabilize it in the apical domain (Nunes de Almeida, 2019).

This work suggests that the Par complex is able to get to the cortex through interacting with either Cdc42 or Par3, but proper polarization is reliant on Cdc42 specifically. The authors interpret their data to suggest that interaction with Par3 initial localizes the Par complex which is then transferred to Cdc42 in the apical domain and further stabilized by interacting with Crb (Nunes de Almeida, 2019). This is a very similar model to the cycling model present in *C. elegans* polarity discussed above.

### *Mammalian epithelia*

Epithelia in mammals relies on many of the same molecular components as in *Drosophila*, including the Par complex, Par3, and Cdc42. These cells form apical

domains that are separated from the basolateral sides by tight junctions (Goldstein and Macara, 2007).

Both Cdc42 and Par3 appear to be required for Par complex apical localization in mammalian epithelia (Hao et al, 2010; Martin-Belmonte et al., 2007; McCaffrey and Macara, 2009). In MDCK cyst formation, knock down of either Cdc42 or Par3 kept aPKC from localizing to the apical domain (Hao et al., 2010; Martin-Belmonte et al., 2007). This was also seen for knock down of Par3 in mouse mammary gland epithelia. Interestingly expression of a Par3 splice variant that does not contain the APM region led to its lateral expansion in these cells and was able to bring aPKC along with it (McCaffrey and Macara, 2009). This data supports the idea that Par3 may have other interactions with aPKC (see Par Complex Interactions above).

Additionally in MDCK cells, it was shown that the apical recruitment of Cdc42 relies on Annexin2, which is recruited by apical enrichment of $PIP_2$ (Martin-Belmonte et al., 2007). However how this recruitment occurs was not determined.

Since both of these proteins are required, it is tempting to suggest that a similar "hand off" between Par3 and Cdc42 of the Par complex that has been proposed for other systems (Nunes de Almeida et al., 2019; Rodriguez et al., 2017) may be occurring in these tissues (McCaffrey and Macara, 2009).

## CURRENT OVERALL MODEL OF PAR COMPLEX REGULATION

From these and other systems, a model emerges whereby the Par complex requires Par3 and/or Cdc42 for cortical targeting and apical polarization. In many contexts, Par3 or Cdc42 appears to be dispensable for the initial cortical targeting,

although they both appear to have a role in fine-tuning or stabilizing localization or in regulating activity once at the membrane (Goldstein and Macara, 2007; Lang and Munro, 2017). In *C. elegans* zygotes and epithelia from *Drosophila* and mammals, this has led to the support of a cycling model, where Par3 may serve to initially recruit the Par complex, but it must then hand it off to Cdc42 (or some other apical protein) whether for localization to the apical domain in epithelia or for activation in *C. elegans* (McCaffrey and Macara, 2009; Nunes de Almeida, 2019; Rodriguez et al., 2017). However this does not seem to always be the case, as can be illustrated by the apparent non-redundant requirement for Cdc42 and Par3 in *Drosophila* neuroblasts (Atwood and Prehoda, 2007).

What can be taken away from all of this data is that Par3 &/or Cdc42 is required in all of these systems for cortical targeting &/or polarization of the Par complex.

**Are there phenotypes that are not explained by this model?**

What about the instances in the literature where aPKC is depolarized (i.e. cortical/expanded on cortex)? Do these contexts rely on these same regulators or are we missing some interaction to explain these phenotypes?

There are many examples of this phenotype in the *Drosophila* polarity literature. It has been shown that mutation of Dap160 (Dynamin-associated protein 160) causes aPKC to become depolarized and inactive as it can colocalize with its substrates on the cortex (Chabu and Doe, 2008). Mutation of AuroraA the cell cycle regulatory kinase has been shown to cause depolarization of aPKC in a subset of neuroblasts. aPKC is also likely to be inactivated in this mutant as a subset of neuroblasts also showed depolarized Numb (Lee et al., 2006a). Mutations within two different components of PP2A

(Microtubule star, the catalytic subunit, and Twins, a regulatory subunit) lead to depolarized aPKC but have varying effects on activity (Chabu and Doe, 2009; Wang et al., 2009a). Treatment of neuroblasts with Latrunculin-A to disrupt cortical F-actin leads to cortical aPKC (Oon and Prehoda, 2019). Additionally disruption of the negative regulator Lgl either by mutation or expression of a non-phosphorylatable version (Lgl3A) also leads to cortical aPKC (Atwood and Prehoda, 2009; Lee et al., 2006b).

Can these different contexts be explained by ectopic localization/interactions with Cdc42 and Par3 or is the model missing other regulatory interactions?

**What about the role of aPKC activity?**

Recently groups working in both *C. elegans* embryos and *Drosophila* neuroblasts showed that disruption of aPKC kinase activity either through genetic manipulation, chemical inhibition or both leads to depolarized, cortical aPKC. In the *C. elegans* paper, Par3 was dispensable for this localization but Cdc42 was required at least during the maintenance phase. Cdc42 RNAi still showed expanded aPKC during establishment (Rodriguez et al., 2017). Was this just due to incomplete penetrance with the RNAi or is something else required to initially localize aPKC to the cortex in this context and Cdc42 is required for maintaining it there later? In the *Drosophila* paper, cortical aPKC brought Par6 to the cortex as well, but Par3 was unperturbed (Hannaford et al., 2019). Does this interaction require Cdc42 or could something else explain this phenotype?

**KNOWLEDGE GAP**

Although the polarity field generally agrees that aPKC targeting to specific regional domains requires Par3 and/or Cdc42 depending on the specific cellular context, this model does not explain what is happening when aPKC is depolarized as has been seen in a variety of mutational contexts, including disruption of aPKC's own kinase activity. It is still unclear whether these instances of aPKC localizing to regions outside of the apical (or anterior) domain relies on these known upstream regulators or if some other interaction is missing from the current model.

Additionally how Par3 interacts with aPKC remains confusing as there are at least 5 purported interactions between them currently described in the literature. It remains to be seen which of these interactions are actually required for Par3 recruitment of aPKC, and what the function of the others are.

Since we ultimately want to know what the direct, specific interactions are that lead to Par complex and therefore aPKC polarization, these areas of research need to be addressed.

## BRIDGE TO CHAPTER II

In this chapter, I have focused on laying out the background and history of Par complex polarization and the known specific interactions with the upstream regulators Par3 and Cdc42, leading to the current model of aPKC recruitment and polarization on the membrane. This model however does not take into account a variety of mutational backgrounds where aPKC is depolarized. Specifically multiple, recent papers have shown that kinase inactive mutants of aPKC have depolarized localization. In *C. elegans*, this was attributed to relying on Cdc42 for localization; however, it is not known whether this

is also the case in other instances. What the role of aPKC activity is in polarization is unknown. In Chapter II, I describe data that shows an additional interaction of aPKC's C1 domain with the lipids of the plasma membrane is required for aPKC polarization in *Drosophila* neuroblasts. Additionally the C1 domain of aPKC is a general cortical recruitment motif that is able sufficient to get to the cortex and must be autoinhibited by intramolecular interactions within aPKC itself. This adds a third, direct interaction that aPKC must have for polarization.

# CHAPTER II

# PROTEIN-LIPID AND PROTEIN-PROTEIN INTERACTIONS COOPERATE TO POLARIZE THE PAR COMPLEX IN DROSOPHILA NEUROBLASTS

*This chapter contains previously unpublished co-authored material

Author contributions: K.A.J. generated flies and performed the analysis. M.L.D. generated flies and performed initial analysis. K.E.P, K.A.J., and M.L.D. were involved in the experimental design. K.A.J. wrote the manuscript. K.E.P directed the research.

## INTRODUCTION

Animals cells rely on cell polarization for proper function and inherited fates of their cells during development and homeostasis. They achieve this through use of the evolutionarily conserved Par complex consisting of the PDZ domain, scaffold protein Par6 and atypical Protein Kinase C (aPKC). This complex is used by a variety of cell types and organisms across metazoan animals to polarize (Lang and Munro, 2017).

The Par complex, which localizes asymmetrically in cells, functions by displacing substrates from its cortical domain, thereby setting up a polarized distribution of proteins. This function is carried out by the kinase activity of aPKC whereby substrates containing phosphorylation sites within a basic-hydrophobic motif that allows them to localize to the cell cortex are removed by addition of negative charge to this motif through phosphorylation, effectively disturbing charged-based interactions with the negatively-charged phospholipids of the cell cortex (Bailey and Prehoda, 2015; Dong et al., 2015).

As this activity can be very powerful and can have drastic consequences if not controlled properly, including gross morphology and tissue misregulation, the regulation of aPKC appears to be tightly controlled (Reina-Campos et al., 2019; Wirtz-Peitz et al., 2008). Two key upstream regulators of the Par complex, the multi-PDZ domain protein Par3 (also known as Bazooka in *Drosophila*) and the small GTPase Cdc42, are used in a variety of cell types to regulate both the specific localization of the Par complex and the activity of aPKC. The literature proposes a model where the Par complex relies on Par and/or Cdc42 for localization across cell types that utilize the Par complex for polarity (Lang and Munro, 2017). However there are a variety of mutational backgrounds where aPKC is depolarized that have not been addressed using this model (Atwood and Prehoda, 2009; Chabu and Doe, 2008; Chabu and Doe, 2009; Lee et al., 2006a; Lee et al., 2006b; Oon and Prehoda, 2019; Wang et al., 2009a). The model also does not take into account what role aPKC's own kinase activity may have in its localization.

Recently groups working in two different polarity systems, *Drosophila* neuroblasts and *C. elegans* one-cell embryos, showed that inactivating aPKC kinase activity through either genetic mutations or chemical inhibition leads to its cortical localization (Hannaford et al., 2019; Rodriguez et al., 2017). Specifically in *C. elegans* one cell embryos, it was shown that the cortical localization of kinase inactive aPKC was not reliant on Par3 but appeared to rely on Cdc42. A closer look at the data does not give quite so clear a picture, as the mutant kinase still appeared to localize on the anterior cortex and expanded into the posterior domain during polarity establishment (Rodriguez et al., 2017). This could suggest that additional interaction serve to initially localize aPKC to the cortex.

In this work, we sought to determine whether kinase inactive aPKC relies exclusively on upstream regulators for its localization or if some unknown interaction was required.

## RESULTS

### A kinase inactive mutant of aPKC has depolarized localization

To test the how kinase regulates aPKC localization in *Drosophila* neuroblasts, we created MARCM larval neuroblast clones expressing HA-tagged aPKC constructs in an *apkc*$^{K06403}$ null background (Lee and Luo, 1999). These *apkc*$^{K06403}$ null clones contain no endogenous aPKC by third instar wandering larval stage, as the basal fate determinant Miranda (Mira) is completely cortical (Figure 1A). We had previously shown expression of a full length HA-tagged aPKC construct (aPKC FL 1-606) was apically polarized in metaphase *apkc*$^{K06403}$ null clones. These clones also had basal crescents of Mira, showing that the construct was active (Holly et al., 2020, this work Chapter III).

For this work, we created an HA-tagged aPKC kinase inactive mutant that disrupts coordination of the γ-phosphate of ATP, aPKC D388A (Cameron et al., 2009; Holly and Prehoda, 2019). We found that aPKC D388A localized to the entire cortex of *apkc*$^{K06403}$ metaphase null clones, colocalizing with Mira which was also cortical in these cells, confirming the inactivity of this construct (Figure 1A). This data agrees with the cortical localization of kinase inactive aPKC seen in other studies (Hannaford et al., 2019; Rodriguez et al., 2017).

Why does inhibition of aPKC kinase activity disrupt the proper regulation of its localization? One possible model that could explain this phenotype is the reliance of

aPKC on a feedback loop for its polarization. In this model, aPKC would phosphorylate some substrate that then would help to recruit it specifically into the apical domain or conversely would block its recruitment into the basal domain. This model would also predict that reintroduction of endogenous aPKC kinase activity to cells with kinase inactive aPKC constructs should be able to rescue the localization defect and polarize them.

**aPKC polarization does not rely on a feedback loop involving its own activity**

To test if feedback could explain the cortical localization of aPKC kinase inactive mutants, we overexpressed aPKC FL or aPKC D388A in larval neuroblasts containing wild-type, endogenous aPKC. We also tested the localization of a well-known kinase inactive mutation K293W, which blocks ATP binding (Graybill et al, 2012). To monitor the location of the transgenes specifically, we stained for HA signal.

We found that aPKC FL was able to polarize to the apical domain in metaphase neuroblasts containing endogenous aPKC (Figure 1B). An increased HA signal was seen in the cytoplasm of these cells, however we believe that this is due to an increase in the cytoplasmic, inactive pool of aPKC, as Mira was still localized to the basal cortex. Even though endogenous aPKC activity was present, aPKC D388A and aPKC K293W localized to the entire cortex in these neuroblasts (Figure 1B). These constructs also colocalized with the basally polarized Mira, showing that they are inactive. To quantify this data, we looked at two metrics: Apical:Cytoplasmic Intensity ratios to measure overall recruitment to the membrane and Apical:Basal Intensity ratios to measure the amount of polarization or depolarization. Both kinase inactive mutants were recruited to

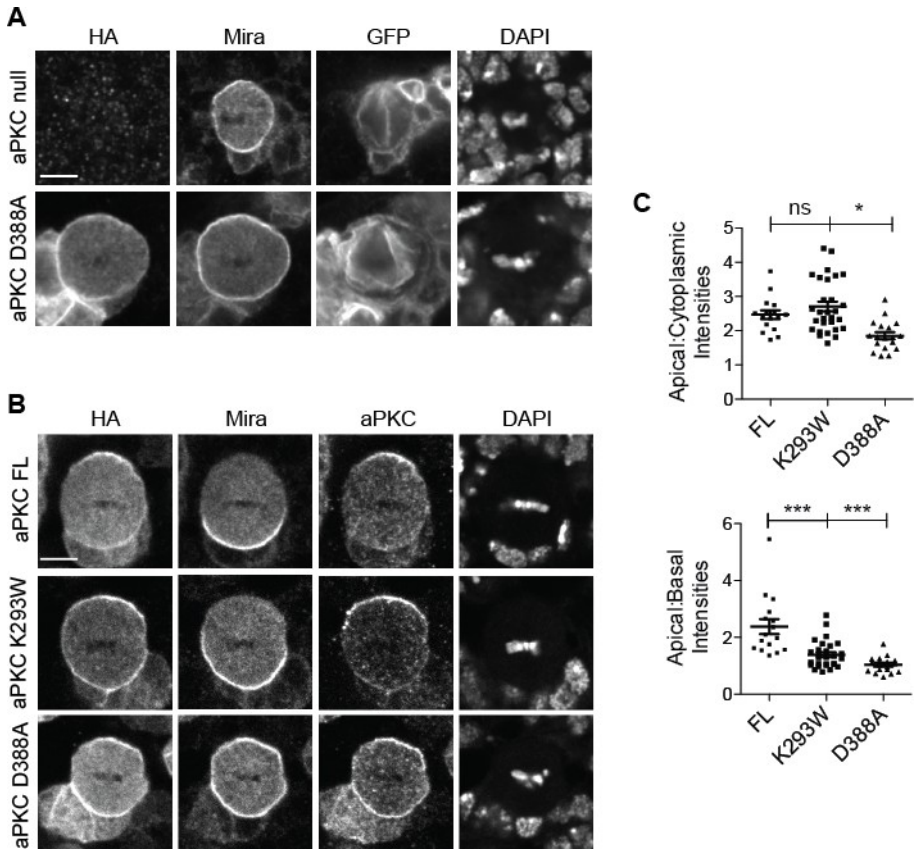


**Figure 1: aPKC activity does not feedback to regulate its polarization.**
(A) HA, Mira, GFP, and DAPI staining of 3xHA-aPKC D388A in *aPKC*$^{K06403}$ MARCM metaphase larval neuroblast clone with *aPKC*$^{K06403}$ null clone as negative control. (B) HA, Mira, aPKC, and DAPI staining of HA-aPKC FL, K293W or D388A in metaphase larval neuroblasts expressed with 1407Gal4/UAS. Note: aPKC antibody marks both endogenous and overexpressed aPKC. (C) Quantification of HA signal in (B). Scale Bars = 5µm.
Statistics in (B): One -way ANOVA with Tukey's post test. * = $p<0.05$, *** = $p<0.0001$.

the cortex, although aPKC D388A appears to be slightly weaker when measuring the apical cortical amount. However this may be explained by the fact that it shows very strong cortical localization with approximately equal amounts of protein in the apical and basal domains. aPKC K293W is also cortical with slightly more apical protein (Figure 1C). Interestingly, the two aPKC mutants had different effects on the endogenous aPKC. aPKC K293W appeared to have no effect on endogenous aPKC, as Mira was still

polarized to the basal domain, indicating active, apical aPKC is present. However aPKC D388A appeared to have a dominant negative effect as Mira showed depolarized localization, often becoming completely cortical (Figure 1B).

**Upstream regulators Cdc42 and Par3 are not required for cortical targeting of kinase inactive aPKC**

It has been shown that the small GTPase Cdc42 is cortically localized throughout the cell cycle in *Drosophila* larval neuroblasts with slight apical enrichment during metaphase. Although it is not currently known whether the entire cortical pool is activated, it is believed that Cdc42 is only active within the apical domain. This assumption comes from *in vivo* studies showing constitutively active Cdc42 is able to recruit both aPKC and Par6 to the cortex and *in vitro* studies which showed Par6 binds preferentially to active (or GTP-bound) Cdc42 (Atwood et al., 2007; Heinrique and Schweisguth, 2003). Par3 on the other hand is apical in metaphase neuroblasts (Atwood et al., 2007).

Hannaford *et al*. showed that their kinase inactive mutant of aPKC was able to bring Par6 along with it onto the entire cortex, but Par3 remained polarized in these cells (Hannaford et al., 2019). This data would suggest that Par3 is not required for the cortical localization of kinase inactive aPKC in *Drosophila* neuroblasts, but perhaps Par6 or Cdc42 working through Par6 is. Interestingly Cdc42 was found to be required for the cortical localization of kinase inactive aPKC in *C. elegans* one cell embryos, although this was only clearly the case in the maintenance phase. During the establishment phase, there appeared to be expanded kinase inactive aPKC still on the cortex. This group also

showed that Par3 was not required, as kinase inactive aPKC remained on the cortex of embryos treated with Par3 RNAi (Rodriguez et al., 2017).

To determine whether our kinase inactive aPKC mutants had an effect on the localization of Par3 or Par6, we stained for these proteins in larval brains expressing our constructs. We found that Par3 remained apically polarized in cells expressing aPKC FL, as well as those expressing aPKC D388A or aPKC K293W, although the intensity of this crescent was slightly reduced in aPKC D388A (Figure 2A, C). As the amount of Baz in wild-type crescents appears to be very variable (data for aPKC FL in Figure 2C), this may be due to low number of cells analyzed for aPKC D388A. Par6 was apically polarized in aPKC FL neuroblasts but was reduced on the cortex and appeared expanded into the basal domain in aPKC D388A and aPKC K293W (Figure 2A, B). Thus cortical kinase inactive aPKC also brings Par6 along with it, but Par3 is still polarized.

Since Par6 appeared to colocalize on the cortex with our kinase inactive aPKC constructs, this could indicate that the cortical localization is reliant on Cdc42 as was seen in the maintenance phase in *C. elegans* (Rodriguez et al., 2017). To test this, we used RNAi to knock down expression of Cdc42 in brains expressing our constructs. As has been previously described, aPKC FL became completely cytoplasmic upon loss of Cdc42 (Atwood et al., 2007) (Figure 2D, E). This was not the case for aPKC K293W, which was able to remain on the cortex even when Cdc42 was absent (Figure 2D, E). We conclude from this data that aPKC K293W is able to localize to the cortex independently of Cdc42.

Although Baz remained polarized in neuroblasts overexpressing kinase inactive aPKC, it is possible that a small amount of Baz could be present in the basal cortex that is

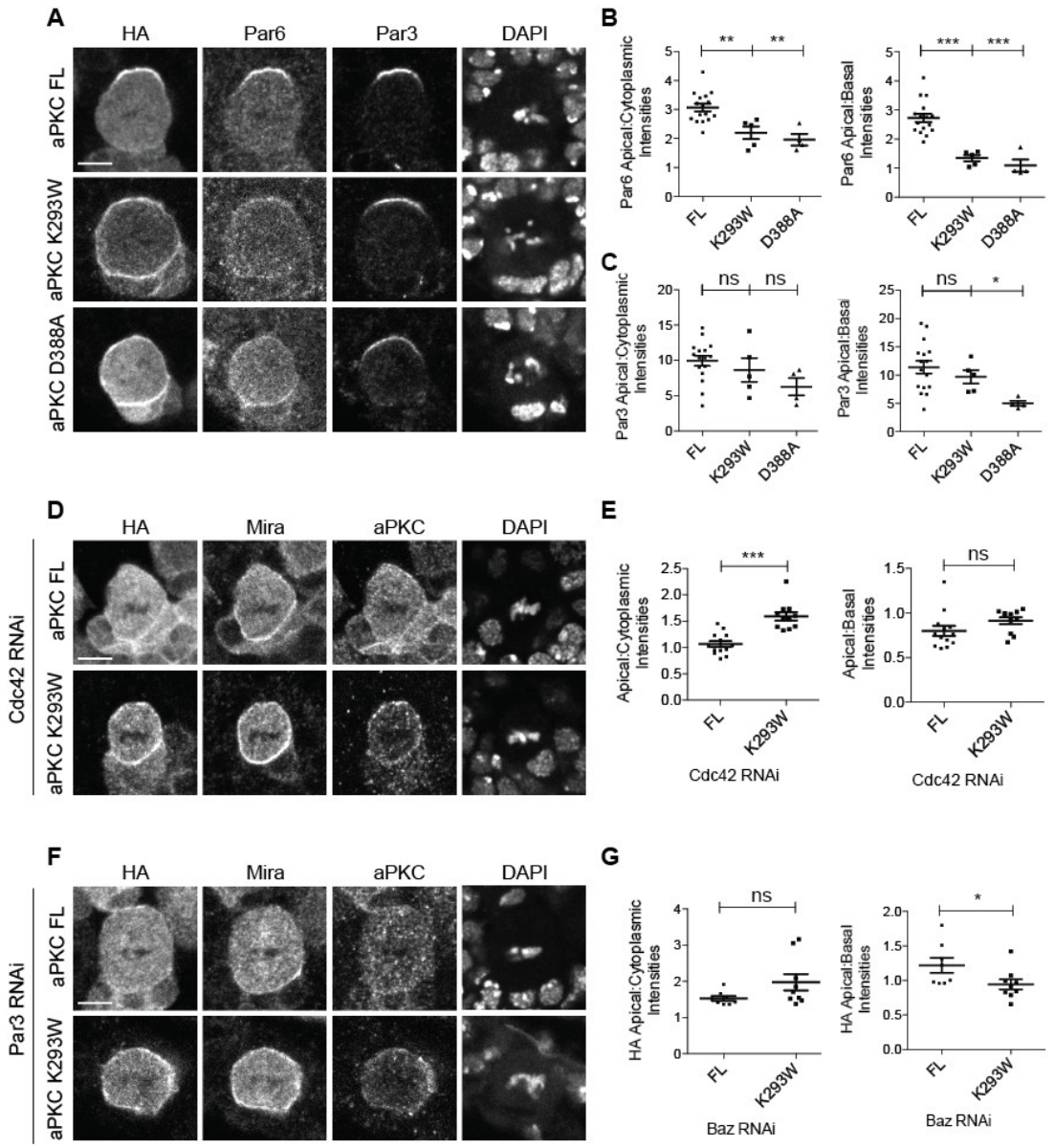


**Figure 2: Kinase inactive aPKC does not appear to rely on upstream regulators Cdc42 or Par3 for its localization.**

(A) HA, Par6, Par3, and DAPI staining of HA-aPKC FL, K293W, or D388A expressed in metaphase larval neuroblasts with 1407Gal4/UAS. (B) Quantification of Par6 signal in (A). (C) Quantification of Par3 signal in (A). (D) Knockdown of Cdc42 using RNAi in metaphase larval neuroblasts expressing HA-aPKC FL or K293W with 1407Gal4/UAS. Stained for HA, Mira, aPKC, and DAPI. aPKC antibody marks both endogenous and overexpressed aPKC (also in (F)). (E) Quantification of HA signal in (D). (F) Knockdown of Par3 using RNAi in metaphase larval neuroblasts expressing HA-aPKC FL or K293W with 1407Gal4/UAS. Stained for HA, Mira, aPKC, and DAPI. (G). Quantification of HA signal in (F). Scale Bars = 5μm.

Statistics in (B): One-way ANOVA with Tukey's post test. Statistics in (E), (G): Two-tailed, unpaired t-test. * = $p<0.05$, *** = $p<0.0001$.

below the detection of our antibody or alternatively that cortical localization of kinase inactive mutants may rely on Baz for initial cortical targeting but then expand along the cortex afterward. To test these hypotheses, we also knocked down Par3 with RNAi. In this context, aPKC FL appears to become more cytoplasmic as expected (Atwood et al., 2007). However it appears that knockdown is incomplete in these cells as some aPKC FL remains in the apical domain (Figure 2F, G). aPKC K293W remained cortically localized when Par3 was reduced (Figure 2F, G). Additionally we believe that Par3 was reduced to a greater extent in these cells, as Mira shows complete cortical localization but was basal when aPKC K293W was overexpressed alone (compare Figure 1B, C to Figure 2F, G). From this data, we conclude that aPKC K293W likely does not rely on Par3 for cortical targeting.

This data shows that the cortical localization seen when disrupting aPKC kinase activity in *Drosophila* neuroblasts cannot be explained with the current polarity model. aPKC K293W was able to localize to the entire cortex in *Drosophila* larval neuroblasts when either Cdc42 or Par3 were knocked down. This is in contrast to kinase inactive aPKC in *C. elegans* that relied on Cdc42 (at least during the maintenance phase) for its localization (Rodriguez et al., 2017).

**Localization of aPKC kinase inactive mutants is uncoupled from cell cycle control**

In wild-type neuroblasts, the Par complex is cytoplasmic in interphase, only becoming recruited to the apical domain upon entry into mitosis. Its localization then returns to the cytoplasm once cytokinesis is completed and the subsequent interphase stage has begun (Oon and Prehoda, 2019).

Upon examining the brains of *Drosophila* expressing aPKC D388A or aPKC K293W, we noticed that these mutants appeared to show cortical localization throughout the cell cycle, even often localizing to the cortex in interphase. This was not seen aPKC FL, which remains cytoplasmic in interphase. (Figure S1A).

**What other functions does the kinase domain have?**

The main function of the kinase domain of aPKC is to phosphorylate substrates. However in kinase inactive mutants, this function is ablated. What other functions does the kinase domain have? aPKC's kinase domain is thought to have intramolecular interactions with at least two domains within its n-terminal regulatory region: the pseudosubstrate and the C1 domain.

The pseudosubstrate mimics the aPKC substrate recognition motif except it contains an alanine in the place where a phosphorylatable serine would be. This domain is known to bind into the catalytic cleft of aPKC, effectively blocking of substrates from occurring. Part of the activation mechanism of aPKC is thought to involve the removal of this motif from the catalytic cleft, possibly through the binding of Par6 (Graybill et al., 2012)

In a deuterium exchange assay, the kinase domain of PKCι was shown to be protected in two regions, the catalytic cleft and the exterior region of the αC-helix, which is integral to the catalytic cleft. This protection was determined to be due to the pseudosubstrate binding into the catalytic cleft positioning the C1 domain to protect the exterior region of the αC-helix (Zhang et al., 2014). This same group had previously shown that both the pseudosubstrate and the C1 domain were able to inhibit the kinase

activity of PKCζ through different mechanisms and modeled a similar localization for these domains onto the kinase domain (Lopez-Garcia et al., 2011).

We have shown that feedback involving aPKC activity and regulation by the upstream regulators Cdc42 and Par3 does not explain the phenotype seen in our aPKC kinase inactive mutants. Additionally these kinase inactive mutants localize to the cortex throughout the cell cycle suggesting that their normal regulation has been disrupted. This data leads us to hypothesize that there may be a previously undefined cortical targeting motif within aPKC itself that is exposed by disruption of kinase activity. Given that the kinase domain is able to interact with two domains within its N-terminal regulatory region that have been implicated in regulation of aPKC activity, we sought to test whether either of these domains is involved in cortical targeting or polarization of aPKC.

**The pseudosubstrate of aPKC is not required for polarization but may be required for stabilizing aPKC at the cortex**

To test whether the pseudosubstrate is required for aPKC polarization, we created an aPKC pseudosubstrate mutant containing mutations of the recognition sequence arginines and the alanine (RRGARR to AAGDAA). This should cause the pseudosubstrate to be removed from the catalytic cleft of aPKC rendering it active.

We found that aPKC AADAA was polarized in metaphase larval neuroblasts; however this was significantly less than aPKC FL. We also found that the cytoplasmic pool due to overexpression was active as Mira was completely cytoplasmic in these cells, confirming that the catalytic cleft was available for substrates (Figure 3A, B). From this data, we conclude that the pseudosubstrate is not required for aPKC polarization, but it

may serve a stabilizing function as the polarized aPKC AADAA was less than aPKC FL. Importantly given that aPKC AADAA is still able to polarize, the pseudosubstrate is not likely to be the cortical targeting signal.

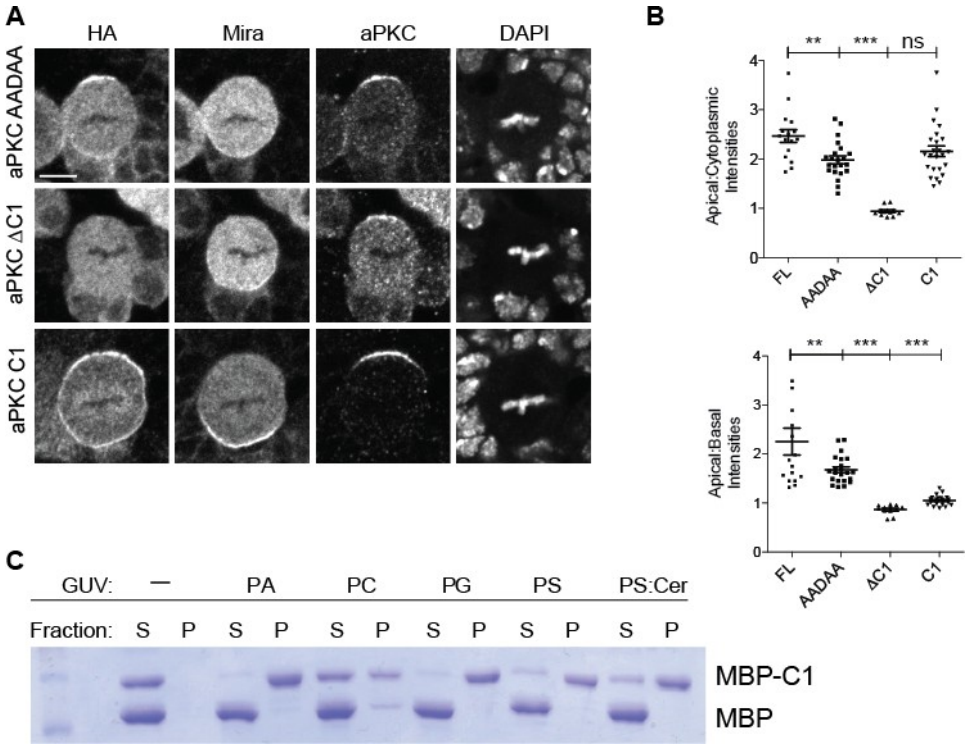


**Figure 3: aPKC C1 domain is sufficient to bind to lipids and is required for aPKC polarization.**
(A) HA, Mira, aPKC, and DAPI staining of HA-aPKC AADAA, ΔC1, or C1 expressed in metaphase larval neuroblasts using 1407Gal4/UAS. aPKC antibody marks both endogenous and overexpressed aPKC. (B) Quantification of HA signal in (A) compared to measurements for HA-aPKC FL from Figure 1. Note: Data point out of range in Apical:Basal Intensities; FL = 5.455764. (C) Lipid pelleting assay using GUVs composed of the following lipids: PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; and Cer, ceramide. PS:Cer GUVs were made from an equal molar ratio of the two lipids. Scale Bar = 5μm.
Statistics in (B): One-way ANOVA with Tukey's post test. ** = $p<0.001$, *** = $p<0.0001$.

**The C1 domain of aPKC is required for polarization**

To determine whether the C1 domain is required for cortical targeting, we created an aPKC construct without the C1 domain (aPKC ΔC1). This construct also contains all known required interaction domains (PB1 domain to bind to the Par6 PB1 domain, PDZ binding motif to bind to the PDZ2 of Par3, and intact activity to phosphorylate substrates). In metaphase neuroblasts, this construct was completely cytoplasmic, but it was partially active as there was a decrease in basal Mira (Figure 3A, B). This data suggests that the C1 domain is required for polarization. Since it is cytoplasmic, this suggests that it may also be required for cortical targeting, but does it act as the cortical targeting signal?

**aPKC C1 domain is sufficient to drive cortical targeting**

C1 domains in conventional and novel PKCs are known to be lipid interacting motifs that bind to diacylglycerol (DAG), partially inserting themselves into the membrane to localize their respective PKCs. However atypical PKC C1 domains contain mutations within the putative DAG binding region that render them incapable of binding to DAG (Pu et al., 2006). The mutations create a charged surface that is no longer able to insert into the membrane as well as changing the size of the cleft that interacts with DAG (Pu et al., 2006). It is these mutations and their inability to bind DAG that labels these C1 domains as atypical and gives the atypical PKCs their name (Suzuki et al., 2003). Due to this difference, aPKC C1 domains have not been well-studied, and their function has remained unknown.

However they have been potentially implicated in the activation mechanism of aPKCs. *In vitro* work has shown that adding lipids to aPKCs has an activating effect, and the C1 domain may be part of this mechanism; although the pseudosubstrate and V5 region have been proposed as well (Ivey et al., 2014; Suzuki et al., 2003; Wang et al., 2009b). It has also been speculated that atypical C1 domains may still have lipid binding abilities, just to other lipids such as phosphatidylinositides (Suzuki et al., 2003). It has also been shown *in vitro* that removal of the C1 domain activates aPKC activity (Graybill et al., 2012).

To determine whether the C1 domain itself is able to localize to the cortex, we expressed the C1 domain alone in neuroblasts. In metaphase neuroblasts, the C1 domain was able to localize to the entire cortex, showing that this domain itself is sufficient for cortical targeting (Figure 3A, B).

As the cortex itself often refers to not just the plasma membrane but also the underlying cytoskeleton and membrane-associated proteins. We used an MBP-C1 domain in a lipid pelleting assay with giant unilamellar vesicles (GUVs) of various lipid compositions to determine if the C1 can bind to lipids. At high speeds the GUVs will pellet and any bound proteins will bind to them, while unbound proteins would remain in the supernatant. For all tested lipids, the C1 domain was able to bind to the GUVs with varying affinities, showing that the C1 can act as a general lipid binding motif (Figure 3C). From these data, we conclude that the C1 domain is sufficient to localize to the cortex and can act as a general membrane binding motif.

Additionally the C1 domain alone is cortical in interphase cells with strong nuclear enrichment (Figure S1B). Nuclear enrichment has been described before in mammalian homologues of aPKC, PKCι and PKCζ when expressed in HeLa cells and an

NLS signal was mapped onto the C1 domain of these proteins (Perander et al., 2001; Seidl et al., 2012). This NLS appears to be conserved in Drosophila and may explain the nuclear signal in these interphase cells. Nuclear enrichment was also seen in aPKC D388A and aPKC K293W, while aPKC FL nuclear levels appear equal to or reduced compared to the cytoplasm (Figure S1A).

**The C1 domain is autoinhibited by intramolecular interactions within aPKC**

We have shown that the C1 domain of aPKC is able to bind to the entire cortex of metaphase neuroblasts. We also found that the C1 alone was able to interact with the entire cortex throughout the cell cycle, as interphase neuroblasts also showed cortical localization. This activity of the C1 to bind to lipids, suggests that it may be regulated within the context of full-length aPKC in an intramolecularly fashion.

***The kinase domain of aPKC inhibits the C1 domain***

As kinase inactive mutants also show cortical localization, we sought to determine if the kinase domain itself may be part of the regulation of the C1 domain. We created an aPKC construct containing just the N-terminal regulatory half of aPKC through the C1 domain (aPKC RD: 1-195). This construct was able to localize to the entire cortex in metaphase neuroblasts, confirming that it is the regulatory half of aPKC containing the C1 domain that serves to target aPKC to the cortex (Figure 4A, B). Since this construct is able to localize to the cortex, it also suggests that the kinase domain serves as a negative regulatory of C1 activity.

To further demonstrate that it is the C1 domain itself within the regulatory region that is responsible for cortical localization, we removed the C1 domain creating aPKC PB1PS (1-141). This construct was completely cytoplasmic confirming that the C1 is responsible for cortical targeting within the regulatory region of aPKC (Figure 4A, B).

As kinase inactive mutants also show cortical localization, we sought to determine if the kinase domain itself may be part of the regulation of the C1 domain. We took

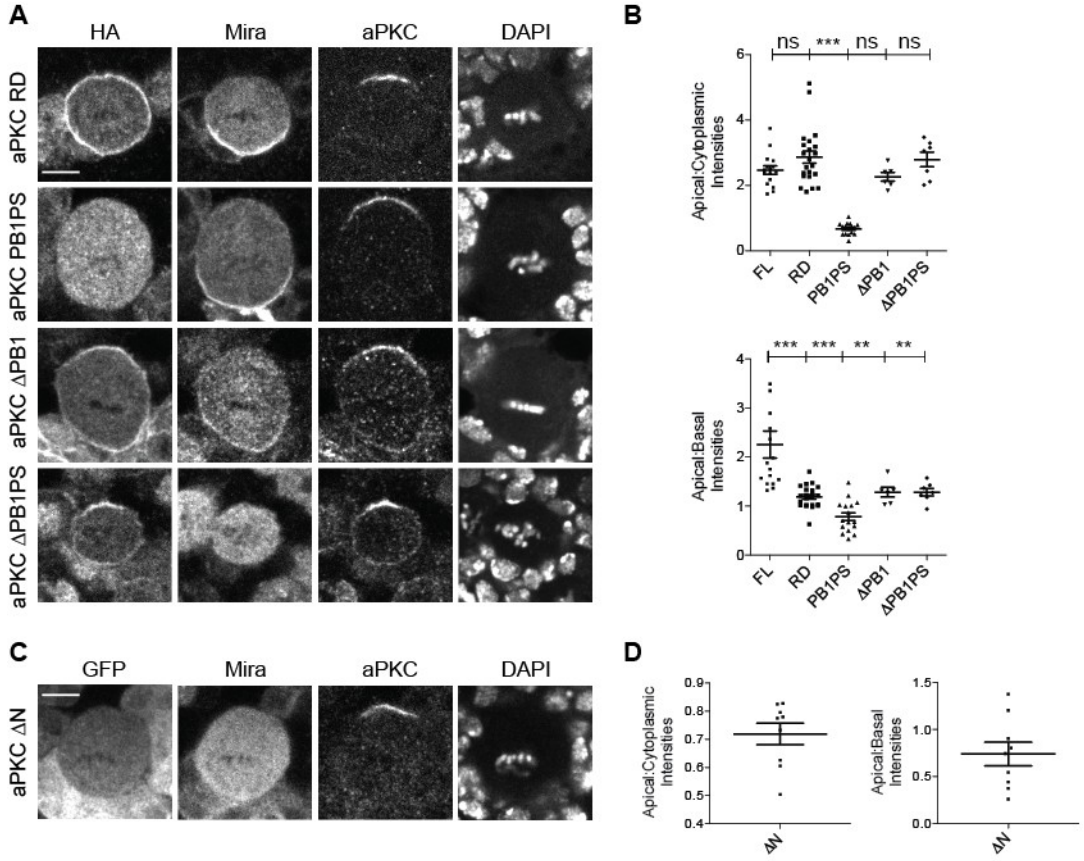


**Figure 4: aPKC C1 domain is regulated by the PB1 and kinase domains.**
(A) HA, Mira, aPKC, and DAPI staining of HA-aPKC RD, PB1PS, ΔPB1, or ΔPB1PS expressed in metaphase larval neuroblasts with 1407Gal4/UAS. aPKC antibody marks both endogenous and overexpressed aPKC (also in (C)). (B) Quantification of HA signal in (A) compared to measurements from HA-aPKC FL in Figure 1. Note: Data point out of range in Apical:Basal Intensities; FL = 5.455764. (C) GFP, Mira, aPKC, and DAPI staining of GFP-aPKC ΔN expressed in metaphase larval neuroblasts with 1407Gal4/UAS. (D) Quantification of GFP signal in (C). Scale Bars = 5μm. Statistics in (B): One-way ANOVA with Tukey's post test. ** = $p<0.001$, *** = $p<0.0001$.

advantage of a known aPKC mutant construct, aPKC ΔN tagged with GFP, which removes the majority of the regulatory domain including all but 15 amino acids of the C1 domain (Lee et al., 2006b). This construct shows completely cytoplasmic localization and is very active, as all Mira has been removed to the cytoplasm. It also has no effect on endogenous aPKC, which is apically polarized (Figure 4C, D). This data shows that the kinase domain itself does not contain a cortical targeting motif. Instead it likely serves to regulate the activity of the C1 domain by inhibiting it.

***The PB1 domain of aPKC inhibits the C1 domain***

Par6 is thought to act as a scaffold for aPKC, as it can interact with both the kinase and Cdc42 through its semi-CRIB domain. The interaction with aPKC relies on heterodimerization between its PB1 domain and that of aPKC (Hirano et al, 2005; Noda et al., 2003). Additionally although the potential interactions for the pseudosubstrate and the C1 domain have been modeled and mapped onto the kinase domain, where or whether the PB1 domain interactions intramolecularly within the context of full-length aPKC is unknown. Indeed in their deuterium exchange assay, Lopez-Garcia et al. only saw two regions of protection in the full-length aPKC that were explained as the pseudosubstrate and C1 interactions sites (Lopez-Garcia et al., 2011). Perhaps the PB1 does not directly interact with the kinase domain itself but may interact with the C1 domain.

To test whether the PB1 domain has any role in regulating the C1 domain, we removed the PB1 domain from aPKC. We found that aPKC ΔPB1 was able to localize to the entire cortex in metaphase larval neuroblasts (Figure 4A, B). This construct was also

cortical and nuclear in interphase cells suggesting that the C1 domain and its NLS are exposed. This same phenotype was seen when we also removed the pseudosubstrate (aPKC ΔPB1PS) confirming that the pseudosubstrate is not required for cortical targeting. aPKC ΔPB1PS is also active as Mira is removed to the cytoplasm (Figure 4A, B).

We conclude that the PB1 domain acts a negative regulator of the C1 domain. Since deletion of either the PB1 or the kinase domain led to cortical localization of their respective aPKC constructs, we conclude that both domains serve to non-redundantly negatively regulate the C1 in full-length aPKC.

## DISCUSSION

In the current polarity model Par3 and/or Cdc42 are required to get the Par complex to the membrane and to polarize them (Lang and Munro, 2017). Previous work showing that disruption of kinase activity in aPKC led us to wonder if this current model of polarity is complete.

We found that in *Drosophila* neuroblasts, the cortical localization of kinase inactive mutants is not reliant on Cdc42 or Par3. This suggested that there was a previously undefined membrane targeting motif within aPKC.

We propose that this membrane targeting motif is the C1 domain, which is sufficient to localize to the cortex and appears to be required for aPKC polarization. We also saw that all cortical mutants tested exhibited constitutive cortical localization with their regulation decoupled from the cell cycle. As this localization was also accompanied by enhanced nuclear localization, we propose that this is further evidence that the C1

domain is “exposed” in these constructs, as it has been previously shown that the C1 domain contains an NLS (Perander et al., 2001; Seidl et al., 2012).

Through deletion constructs we further showed that the PB1 and kinase domains appear to be required non-redundantly to inhibit the ability of the C1 domain to interact with the membrane. Therefore intramolecular interactions appear to regulate the C1 domain.

What is the mechanism of C1 domain regulation? A model that may explain this data could be that the C1 domain is not exposed until other protein-protein interactions with Par6 and Par3 have occurred. This would fit with current data that shows aPKC is initially recruited from the cytoplasm to the cortex in a biased, apical manner (Oon and Prehoda, 2019). This model would also give the C1 the role of stabilizing the interactions at the membrane and perhaps ensuring that aPKC is active there, as addition of lipids to aPKC *in vitro* has been shown to have an activating effect (Suzuki et al., 2003). Perhaps in these experiments, the activation occurs because the C1 domain is “undocking” from the kinase as it interacts with the lipids, allowing aPKC to adopt a more active configuration (Lopez-Garcia et al., 2011; Zhang et al., 2014).

Further analysis of the regulation of the C1 domain and whether its requirement for polarization is conserved in other polarity contexts will help to enlighten its function.

Additionally although the pseudosubstrate did not appear to be required for polarization of aPKC, it may have some role in stabilizing the localization at the apical cortex, as mutating it led to decreased apical recruitment. Further work will be needed to understand the role that this domain has in polarization of aPKC.

## MATERIALS AND METHODS

*Drosophila*

Flies were grown at indicated temperatures on standard cornmeal/yeast media. Both male and female larvae were used in this study.

Fly lines used: ;1407-Gal4 (BDSC_8751) ; FRT-G13, aPKC$^{K06403}$/CyO (Gift from C.Q. Doe), and elav-Gal4, UAS-mCD8:GFP, hs:flp; FRT-G13, tubPGal80 (BDSC_5145), Baz RNAi (BDSC_39072), Cdc42 RNAi (VDRC_100794), aPKCΔN:GFP (Gift from C.Q. Doe).

Fly lines created for this work:

3xHA: 3xHA aPKC D388A; HA: HA aPKC FL (1-606); HA aPKC D388A; HA aPKC K293W; HA aPKC AADAA; HA aPKC C1 (139-195); HA aPKC ΔC1 (Δ141-196); HA aPKC RD (1-195); HA aPKC PB1PS (1-141); HA aPKC ΔPB1 (107-606); HA aPKC ΔPB1PS (139-606).

The above constructs of aPKC were cloned into the pUAST attB vector (GenBank: EF362409.1) modified to include an N-terminal 3xHA or 1xHA tag. Integration of the vectors was done using standard Phi-C31 integration into an attP landing site on the third chromosome (attP2) by Rainbow Genetics or BestGene Inc. Positive insertion was determined from the presence of colored eyes after backcrossing to *y,w* stock.

*Drosophila larval neuroblast Immunofluorescence*

For all overexpression crosses, ;1407-Gal4 (BDSC_8751) Virgins were crossed to males containing an aPKC transgene on the third chromosome or an RNAi on the second

chromosome and an aPKC transgene on the third chromosome. Crosses laid in vials for 24 hours (~20˚C) for 24 hours. The resulting embryos were incubated at 30˚C until larvae reached third instar wandering larva stage.

At this point, they were dissected, such that the tissue was not sitting for longer than 20' before going into 4% PFA fixative for 20'. This and all subsequent wash steps involved agitation by placing on a nutator. After fixation, brains were washed 1xquick, and 3x15' in PBST (1xPBS with 0.3% Triton-X). If brains were not to be stained that day, they would then be placed at 4˚C for up to 3 days after which a quick wash in PBST would be required before moving on to staining. If instead they were to be stained, an additional wash step of 20' in PBST would occur. Brains were blocked for 30' in PBSBT (PBST with 1% BSA) and then put into 1˚ overnight at 4˚C. The next day after removing the 1˚, brains were washed 1xquick and 3x15' in PBSBT and put into 2˚ for 2 hours, protected from light. After 2˚ was removed, brains were washed 1xquick and 3x15' in PBST. Brains were stored in SlowFade w/DAPI at least overnight before imaging.

Primary antibodies: Rat $\alpha\alpha$-Mira (1:500; Abcam, ab197788), Mouse $\alpha\alpha$-PKCζH-1(1:1,000; Santa Cruz Biotech sc-17781), Rabbit $\alpha\alpha$-HA C29F4(1:1,000; Cell Signaling Technologies, 3724), Rt $\alpha\alpha$-Par6 (1:500; our lab), and Guinea Pig $\alpha\alpha$-Baz(1:2,000; gift from C.Doe). Secondary antibodies: Dk $\alpha\alpha$-Rt Cy3 (712-165-153; 1:500), Dk $\alpha\alpha$-Rb 647 (711-605-152; 1:500), Dk $\alpha\alpha$-Ms 488 (715-545-151), Dk $\alpha\alpha$-Gp 488 (706-545-148). Secondary antibodies are from Jackson Immunoresearch.

Brains were imaged on an upright confocal TCS SPE from Leica using an ACS APO 40x 1.15 NA Oil CS objective.

*MARCM Immunofluorescence*

To create MARCM larval neuroblasts clones, FRT-G13, aPKC$^{K06403}$/CyO (Gift from C.Q. Doe) Virgins were crossed with 3xHA aPKC D388A males. The subsequent progeny were allowed to grow to adulthood and were screened for the absence of the CyO marker. Males with no CyO were then crossed to elav-Gal4, UAS-mCD8:GFP, hs:flp; FRT-G13, tubPGal80 (BDSC_5145) Virgins.

Crosses laid in vials for 24 hours and the resulting embryos were incubated at room temperature (~20˚C) for 24 hours. These vials were then heat shocked at 37˚C for 90 min. Another heat shock was possible within 18 hours. Larvae were allowed to grow at room temperature or 18˚C until third instar wandering larva stage, when they were dissected and fixed as above.

Primary antibodies: Rat $\alpha\alpha$-Mira (1:500; Abcam, ab197788), Mouse $\alpha\alpha$-HA (1:500; Covance, MMS-101P), and Chicken $\alpha\alpha$-GFP (1:500; Abcam, ab13970). Secondary antibodies: Dk $\alpha\alpha$-Rt Cy3 (712-165-153; 1:500), Dk $\alpha\alpha$-Ms 647 (715-605-151; 1:500), and Dk $\alpha\alpha$-Ck 488 (703-545-155). Secondary antibodies are from Jackson Immunoresearch.

Brains were imaged using a Fluoview F1000 upright confocal from Olympus with a PlanApo N 60x 1.42 NA Oil objective

*Neuroblast Quantification*

Took measure of a 10px line through the central slice of a neuroblast from apical to basal cortex. Apical was taken as the peak corresponding with the apical membrane and basal was taken as the peak corresponding with the basal membrane. Where no peak was present, peaks in other channels or the “edge” of signal before it dropped was used.

Cytoplasmic signal was taken as the average of 20 data points located 10 points from the apical peak.

All images were analyzed using Fiji and statistical analysis was done in Prizm.

All figures were put together using Adobe Illustrator.

*MBP-C1 protein purification*

MBP-C1 was purified according to standard MBP purification protocols in our lab as previously described (Graybill et al., 2012).

*GUV Preparation*

Lipids were resuspended to 10mg/mL in chloroform (or purchased at this concentration). 50µL of 10mg/mL of each lipid (or lipid combination: PS and ceramide were mixed to molar equivalence) was dried in a test tube under an $N_2$ stream. These were then allowed to dry further in a vacuum chamber to ensure all chloroform was removed. Lipids were resuspended in a 0.2M sucrose solution to a final concentration of 0.5mg/mL and heated in a water bath at 50˚C for 5 hours with occasional agitation. All lipids were stored at 4˚C and used within 3 days.

*Lipid pulldown assay*

For this assay, 10X Dilution Buffer was made: 200mM HEPES, pH 7.5; 500mM NaCl, 10mM DTT. All spins were carried out using an Optima MAX-TL Ultracentrifuge with a TLA-100 rotor at 65,000 at 4˚C.

MBP-C1 protein was diluted to 50μM in 1xDilution Buffer and pre-cleared for 30'. Reaction conditions: 20mM HEPES, pH 7.5, 50mM NaCl, 1mM DTT, 0.25mg/mL GUVs, and 5μM MBP-C1. Reaction was carried out at room temperature for 15' and then spun down for 30'. The supernatant fraction was removed, and the pellet was resuspended in an equivalent volume of 1X Dilution buffer. Both the supernatant and pellet samples were mixed with 6x loading dye and run on a 12.5% SDS-Page gel. Gels were stained with Coomassie and imaged using a scanner.

Lipids from Avanti Polar Lipids: 840032C L-α-phosphatidylserine, 840051C L-α-phosphatidylcholine, 840101C L-α-phosphatidic acid, 841138P L-α-phosphatidylglycerol, and 860512P C12 Ceramide.

**Bridge to Chapter III:**

In this chapter, we showed that aPKC contains a previously uncharacterized general lipid targeting motif, the C1 domain, which is required for aPKC polarity in *Drosophila* larval neuroblasts. Through our data, we also suggest that this domain must be regulated as our mutants show ectopic membrane (and in interphase, membrane and nuclear) localization when the C1 domain is "exposed". In Chapter III, we add to the list of specific, direct interactions that are required for aPKC polarization by narrowing down the cloudy field of aPKC/Par3 interactions to show that only one: a novel interaction between the PDZ Binding Motif (PBM) at the C-terminus of aPKC and the PDZ2 domain of Par3 is required for aPKC polarization. This work using *in vitro* biochemistry and *in vivo* work in *Drosophila* larval neuroblasts helps our understanding of the minimal required components for polarizing aPKC in this system.

# CHAPTER III

# A CONSERVED PDZ-BINDING MOTIF IN APKC INTERACTS WITH PAR-3 AND MEDIATES CORTICAL POLARITY.

*This chapter contains previously published co-authored material

Holly, R.W., Jones, K.A., Prehoda, K.E. (2020). A Conserved PDZ-Binding Motif in aPKC Interacts with Par-3 And Mediates Cortical Polarity. Curr. Biol. *In Press*

Author contributions: R.W.H. performed all *in vitr*o experiments. K.A.J. generated flies and performed all *in vivo* experiments. K.E.P and R.W.H. were both involved in the experimental design. K.E.P and R.W.H. wrote the manuscript. K.E.P directed the research.

## SUMMARY

Par-3 regulates animal cell polarity by targeting the Par complex proteins Par-6 and atypical Protein Kinase C (aPKC) to specific cortical sites. Although numerous physical interactions between Par-3 and the Par complex have been identified (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Renschler et al., 2018; Soriano et al., 2016; Wodarz et al., 2000), we discovered a novel interaction between Par-3's second PDZ domain and a highly conserved aPKC PDZ binding motif (PBM) that is required in the context of the full-length, purified Par-6/aPKC complex. We also found that Par-3 is phosphorylated by the full Par complex and phosphorylation induces dissociation of the

Par-3 phosphorylation site from aPKC's kinase domain but does not disrupt the Par-3 PDZ2 – aPKC PBM interaction. In asymmetrically dividing *Drosophila* neuroblasts, the aPKC PBM is required for cortical targeting, consistent with its role in mediating a persistent interaction with Par-3. Our results define a physical connection that targets the Par complex to polarized sites on the cell membrane.

## RESULTS AND DISCUSSION

### Par complex phosphorylation of Par-3

The catalytic activity of aPKC defines mutually exclusive cortical domains in diverse animal cells (Lang and Munro, 2017; Venkei and Yamashita, 2018). Par-6 and aPKC are recruited to specific cellular sites where aPKC phosphorylation polarizes downstream factors by displacing them from the Par cortical domain. For example, in *Drosophila* neural stem cells or neuroblasts, the Par complex localizes to an apical cortical domain during mitosis where it excludes neuronal differentiation factors (Atwood and Prehoda, 2009; Rolls et al., 2003; Wodarz et al., 1999). Apical exclusion separates these factors into a distinct cortical domain at the basal cortex, which is segregated into the basal daughter cell following cytokinesis (Knoblich, 2010; Venkei and Yamashita, 2018). Par polarized factors such as Miranda and Numb contain sequences that bind the membrane but are also phosphorylation motifs for aPKC (Bailey and Prehoda, 2015). The direct connection of aPKC's catalytic activity to the polarization of downstream factors makes the regulatory pathways that control its cortical targeting critical to animal cell polarity.

In many cellular contexts, Par-3 (Bazooka in flies) is essential for recruitment of Par-6 and aPKC to specific cortical sites (Lang and Munro, 2017; Venkei and Yamashita, 2018; Wen and Zhang, 2018). Par-3's role in regulating Par complex cortical recruitment is thought to be direct because five physical interactions have been discovered with both Par-6 and aPKC (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Renschler et al., 2018; Soriano et al., 2016; Wodarz et al., 2000) (Figure 5A). Four of the interactions involve at least one of Par-3's three PDZ protein interaction domains: Par-3 PDZ1 binding to the Par-6 PDZ domain (Joberty et al., 2000; Li et al., 2010b; Lin et al., 2000),

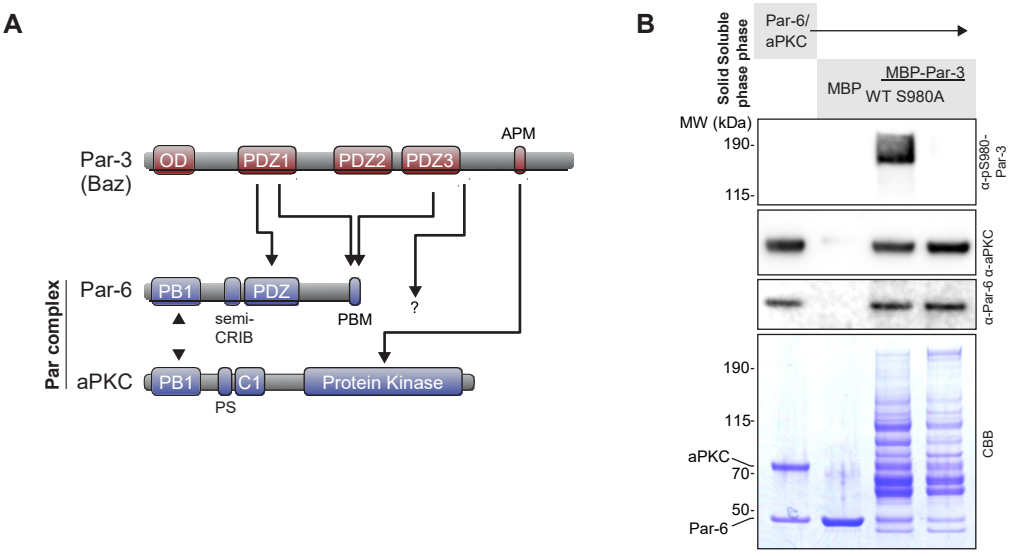


**Figure 5: Par-6/aPKC binds and phosphorylates Par-3.** (A) Domain structure (not to scale) and previously described Par-3 interactions with the Par complex. Single direction arrows define the five previously identified Par-3 interactions with Par-6/aPKC. PBM, PDZ Binding Motif; APM, aPKC Phosphorylation Motif (aka CR3); PS, pseudosubstrate. Double headed arrow denotes the interaction between Par-6 and aPKC. (B) Par-3 interaction with and phosphorylation by the Par complex. Solid phase (amylose resin) bound Maltose Binding Protein (MBP) fused Par-3 (MBP-Par-3 has an expected mass of 199.9 kDa) with the Par-6/aPKC complex. CBB, Coomassie Brilliant Blue; α-pS980-Par-3, antibody specific to the S980 site within the APM [17]. Shaded region indicates fraction applied to gel (soluble phase or solid phase components after mixing with soluble phase components and washing).

Par-3 PDZ1 and PDZ3 domain interactions with Par-6's PDZ Binding Motif (PBM) (Renschler et al., 2018), and an interaction with an undefined region of aPKC that requires both Par-3 PDZ2 and PDZ3 (Wodarz et al., 2000). Additionally, because Par-3 is an aPKC substrate, the aPKC kinase domain interacts with Par-3's aPKC Phosphorylation Motif (APM aka CR3). Although protein kinases are typically thought to interact transiently with their substrates, the interaction with the APM has been proposed to mediate complex assembly (Izumi et al., 1998; Nagai-Tamai et al., 2002; Soriano et al., 2016). Previous investigations used small fragments of the Par complex that did not contain all potential binding motifs, such that it was not possible to assess whether any of the interactions are required for binding in the context of the purified, full-length Par-6/aPKC complex. Furthermore, none of the interactions have been shown to be required for cortical targeting of aPKC in a functional context.

We investigated Par-3 interactions with the Par-6/aPKC complex by reconstituting full-length *Drosophila* Par-6 and aPKC. While we were able to purify the Par-6/aPKC complex to a high degree (Figure 5B), Par-3 is very large (157.4 kDa) and the Maltose Binding Protein fused Par-3 (MBP-Par-3; total mass 199.9 kDa) we were able to obtain included significant amounts of degradation products in addition to full-length protein. Nevertheless, using this preparation we were able to detect an interaction with reconstituted Par complex using a qualitative affinity chromatography (i.e. "pull-down") assay (Figure 5B). Additionally, we detected phosphate transfer to full-length Par-3 (and some smaller fragments with masses consistent with COOH-terminal truncations that contain the APM) using an antibody specific to the phosphorylated APM (Morais-de-Sá et al., 2010) (Figure 5B). Phosphorylation of Par-3 by aPKC has been

controversial (Holly and Prehoda, 2019; Soriano et al., 2016; Thompson and McDonald, 2019). This result contributes to our understanding of the process by demonstrating that aPKC phosphorylates Par-3 in the context of the full-length, purified Par complex in addition to the isolated catalytic domain and APM peptide (Holly and Prehoda, 2019).

**Par-3 PDZ2 is required for interaction with the Par complex**

Using the system of purified Par complex and MBP-fusions of full-length Par-3 and its degradation products, we attempted to identify Par-3 domains required for interaction with the full Par complex (Figure 6A). We also tested the Par-6 PBM within the Par complex as it has been reported to bind both the Par-3 PDZ1 and PDZ3 domains (Renschler et al., 2018) (Figure 6B). We included ATP in binding experiments since Par-

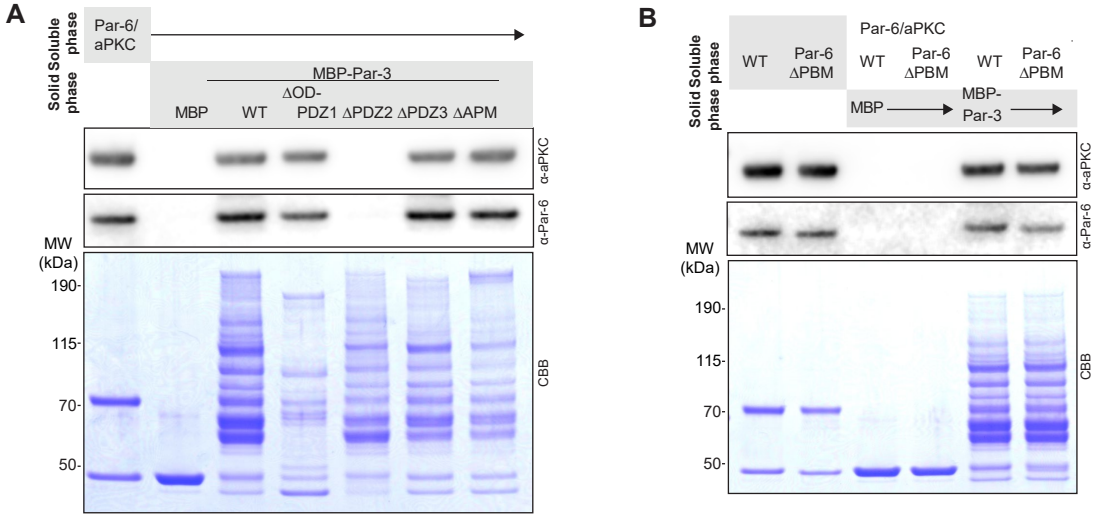


**Figure 6: Par-3 PDZ2 is required for Par complex binding.** (A) Effect of removing Par-3 domains on its interaction with the Par complex. Solid phase (amylose resin) bound Maltose Binding Protein (MBP) fused Par-3 (full length or the PDZ1-APM fragment) was incubated with soluble Par-6/aPKC complex. CBB, Coomassie Brilliant Blue. Shaded region indicates fraction applied to gel (soluble phase or solid phase components after mixing with soluble phase components and washing). (B) Effect of removing the Par-6 PDZ Binding Motif on the Par complex interaction with Par-3. Labeling as in A.

3 is a substrate in the context of the full Par complex (Figure 6B). Using this experimental setup, we identified Par-3 PDZ2 as a required interaction domain for binding to the full Par complex (Figure 6).

**A conserved aPKC PDZ Binding Motif is required for interaction with Par-3**

To determine the mechanism by which Par-3 PDZ2 mediates binding to the Par complex, we first sought to identify the recognition site on the complex. The *Drosophila* aPKC COOH-terminal sequence has the characteristics of a "class 3" PDZ Binding Motif (PBM) (Figure 7A) and is consistent with the binding specificity of the PDZ2 domain as assessed using a phage display assay (Yu et al., 2014). The aPKC COOH-terminal sequence is also highly conserved among metazoan orthologues (Figure 7A), the same evolutionary interval in which Par-3 is found (Fahey and Degnan, 2010). We tested whether the aPKC COOH-terminus is required for the interaction with Par-3 by purifying Par complex lacking aPKC's final six residues. As shown in Figure 7B, the aPKC COOH-terminus is required for Par-3's interaction with the Par complex.

To test whether the Par-3 PDZ2 and aPKC COOH-terminus are sufficient for binding, we examined the interaction of the isolated motifs. As shown in Figure 7C, the isolated proteins are sufficient for complex assembly. In general, PDZ–PBM interactions are strongly dependent on the identity of the terminal residue and we found that Par-3 PDZ2 failed to bind the aPKC COOH-terminus when the final residue was mutated from valine to alanine (aPKC V606A; Figure 7C). We conclude that the aPKC COOH-

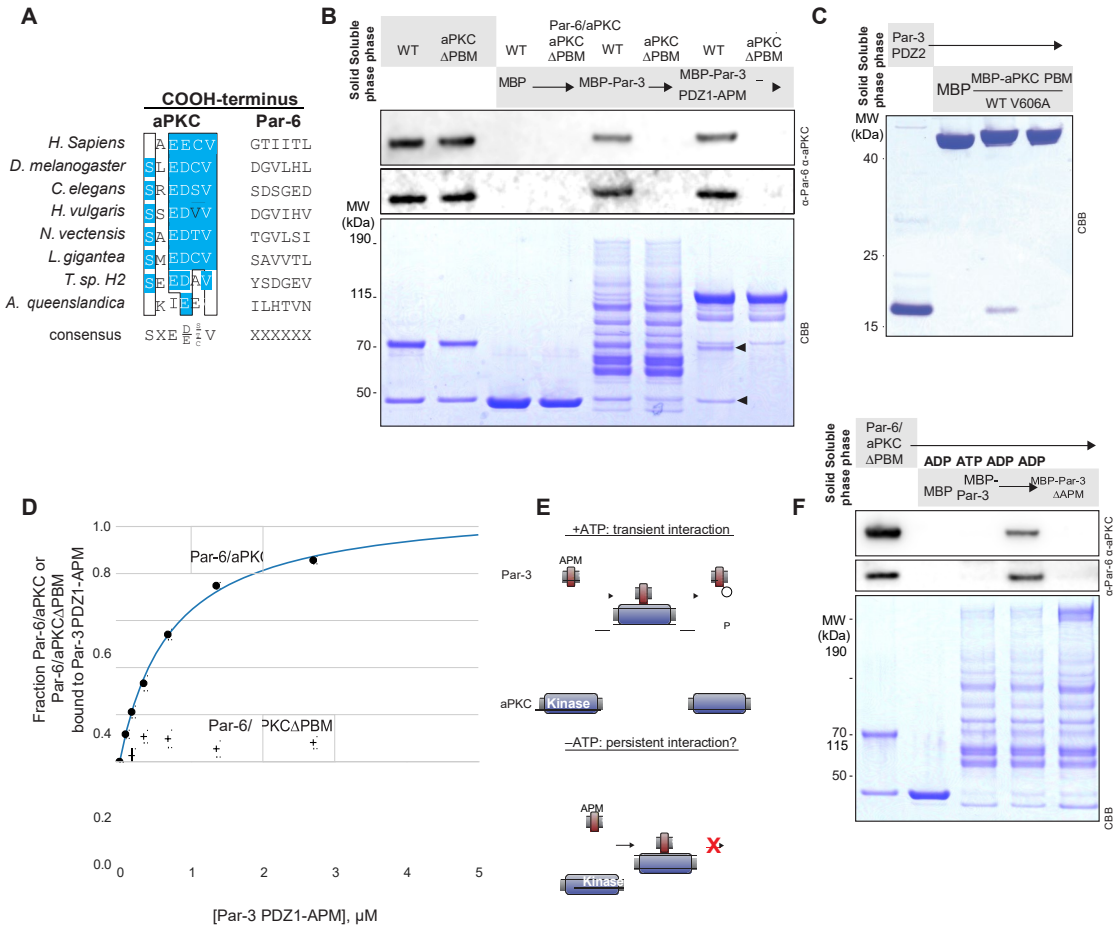


**Figure 7: A conserved aPKC PDZ Binding Motif required for Par-3's interaction with the Par complex.** (A) Sequence alignment of the aPKC and Par-6 COOH-terminuses from diverse metazoan organisms. (B) Effect of removing the aPKC PDZ Binding Motif on the Par complex interaction with Par-3. Solid phase (amylose resin) bound Maltose Binding Protein (MBP) fused Par-3 (full length or the PDZ1-APM fragment that contains all known interaction domains) was incubated with wild type Par-6/aPKC or the complex lacking aPKC's PBM (Par-6/aPKCΔPBM). Arrowheads indicate Par-6 and aPKC. CBB, Coomassie Brilliant Blue. Shaded region indicates fraction applied to gel (soluble phase or solid phase components after mixing with soluble phase components and washing). (C) Interaction of Par-3 PDZ2 with the aPKC PBM. Labeling as in B. For other organisms, see Figure S1A. (D) Comparison of Par-6/aPKC and Par-6/aPKCΔPBM binding to Par-3 PDZ1-APM using an equilibrium supernatant depletion assay [22]. Mean values of the fraction of the Par complex bound, as determined by both anti-Par-6 and anti-aPKC western blot analysis (see Figure S1B), from two experimental replicates are shown along with standard error at each concentration. (E) Schematic depicting how the lack of ATP may convert a transient interaction between the Par-3 APM and the aPKC kinase domain into a persistent one. (F) Effect of nucleotide on the interaction of Par-3 with Par-6/aPKCΔPBM. Solid phase (amylose resin) bound Maltose Binding Protein (MBP) fused Par-3 (full length and with the specified interaction domains removed) was incubated with Par-6/aPKC lacking aPKC's PBM (Par-6/aPKCΔPBM). "ADP" or

"ATP" indicates which nucleotide was present in the binding reaction. CBB, Coomassie Brilliant Blue. Shaded region indicates fraction applied to gel (soluble phase or solid phase components after mixing with soluble phase components and washing).

terminus is a bona fide PDZ Binding Motif (PBM). We also confirmed that the aPKC PBM and Par-3 PDZ2 interaction is broadly conserved across metazoans by examining orthologues from a chordate (human), a placozoan (*Trichoplax*), and a cnidarian (*Hydra*), in addition to the arthropod *Drosophila* (Figure S1A). We observed binding for each of the orthologous pairs indicating that the interaction is conserved across diverse metazoan organisms. Together, these results indicate that the Par-3 PDZ2 and aPKC PBM are sufficient for binding and their interaction is conserved across metazoa.

To assess the role of the Par-3 PDZ2–aPKC PBM interaction quantitatively, we implemented an equilibrium supernatant depletion assay (Pollard, 2010). We measured the affinity of the Par-3 PDZ1-APM for the Par complex, as this region could be purified to a level suitable for quantitative measurements (Figure 7B). Addition of Par-3 PDZ1-APM depleted Par-6 and aPKC from the supernatant consistent with a $K_d$ of 0.7 μM (95% confidence interval of 0.5 – 0.9 μM; Figures 7D, S1B). To determine the effect of disrupting the Par-3 PDZ2–aPKC PBM interaction on binding affinity, we examined Par-3 PDZ1-APM binding to Par-6/aPKCΔPBM. We did not observe sufficient depletion of Par-6 and aPKCΔPBM by PDZ1-APM to allow fitting to a binding isotherm (Figures 7D, S1B), indicating the absence of the aPKC PBM substantially decreases the affinity of the Par-3 interaction with the Par complex, consistent with the results of qualitative measurements (Figure 7B).

**The role of Par-3 phosphorylation in its interaction with the Par complex**

Our results indicate that the Par-3 PDZ2 and aPKC PBM are required for Par-3's interaction with the Par complex (Figures 6A, 7B). The requirement for these domains

suggests that the Par-3 phosphorylation site (i.e. APM) does not form a persistent interaction with the Par complex. However, this conclusion appears to be in conflict with previous work showing that the Par-3 APM is sufficient for binding to the aPKC kinase domain, both with binding assays and structure determination using x-ray crystallography (Soriano et al., 2016; Wang et al., 2012). Furthermore, a stable APM-kinase interaction forms the basis of a model in which the unphosphorylated Par-3 APM forms a stable, persistent interaction with the aPKC kinase domain that is not phosphorylated until an unknown activating event occurs (Soriano et al., 2016). The finding that Par-3 is phosphorylated by the full Par complex (Figure 5B) is inconsistent with this model, but it does not fully resolve whether the Par-3 APM is sufficient for forming a stable, persistent interaction with the Par complex (Soriano et al., 2016; Wang et al., 2012).

We hypothesized that the presence of ATP could influence the binding behavior of the Par-3 APM with the Par complex. A key difference between our experiments and previous reports is that our experiments included ATP, whereas previous binding experiments and structural analysis lacked ATP (Soriano et al., 2016; Wang et al., 2012). Without ATP, completion of the protein kinase catalytic cycle is not possible, and interactions that would otherwise form transiently could persist (Figure 7E).

We tested whether the Par-3 APM forms a stable, persistent interaction with the Par complex in the absence of ATP. We were able to detect binding between Par-3 and the Par complex after replacing ATP with ADP in a context where the Par-3 PDZ2 – aPKC PBM interaction is disrupted (Figure 7F). This interaction requires the APM, leading us to conclude that the Par-3 APM can form a persistent interaction with the Par complex, but only in the absence of ATP. When ATP is present the APM interacts

transiently with the Par complex, because it is phosphorylated (Figure 5B) and subsequently dissociates (Figures 7B, D, F). Under the same conditions, the Par-3 PDZ2 interaction with the Par complex is not disrupted, however (e.g. Figure 7B, D). Although it is possible to form a stalled complex between the Par-3 APM and the Par complex in the absence of ATP, we propose that persistent binding of the APM to the kinase domain due to the lack of ATP is unlikely *in vivo* because ATP concentrations are high under normal cellular conditions.

**The aPKC PDZ Binding Motif is required for neuroblast polarization**

Although numerous interactions have been identified between Par-3 and Par-6/aPKC (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Renschler et al., 2018; Soriano et al., 2016; Wodarz et al., 2000), none have been demonstrated to be required for cortical targeting of the Par complex. In fact, the interactions of Par-6 with Par-3 have been shown to be dispensable for function (Li et al., 2010, 2010; Renschler et al., 2018). To determine if the Par-3 PDZ2 – aPKC PBM interaction is required for Par complex polarization, we investigated the localization of aPKC harboring the V606A PBM point mutation during neuroblast asymmetric division by expressing aPKC-V606A in larval brain neuroblasts and comparing its localization to that of wild-type aPKC. Consistent with previous observations (Oon and Prehoda, 2019; Rolls et al., 2003), we found that wild-type aPKC is polarized to a cortical crescent around the apical pole at metaphase (Figures 8A, B). In contrast, aPKC-V606A remained in the cytoplasm and was not recruited to the cortex, even though the localization of Par-3 was unaffected (Figures 8A-D). The aPKC-V606A protein also failed to be recruited to the apical cortex in

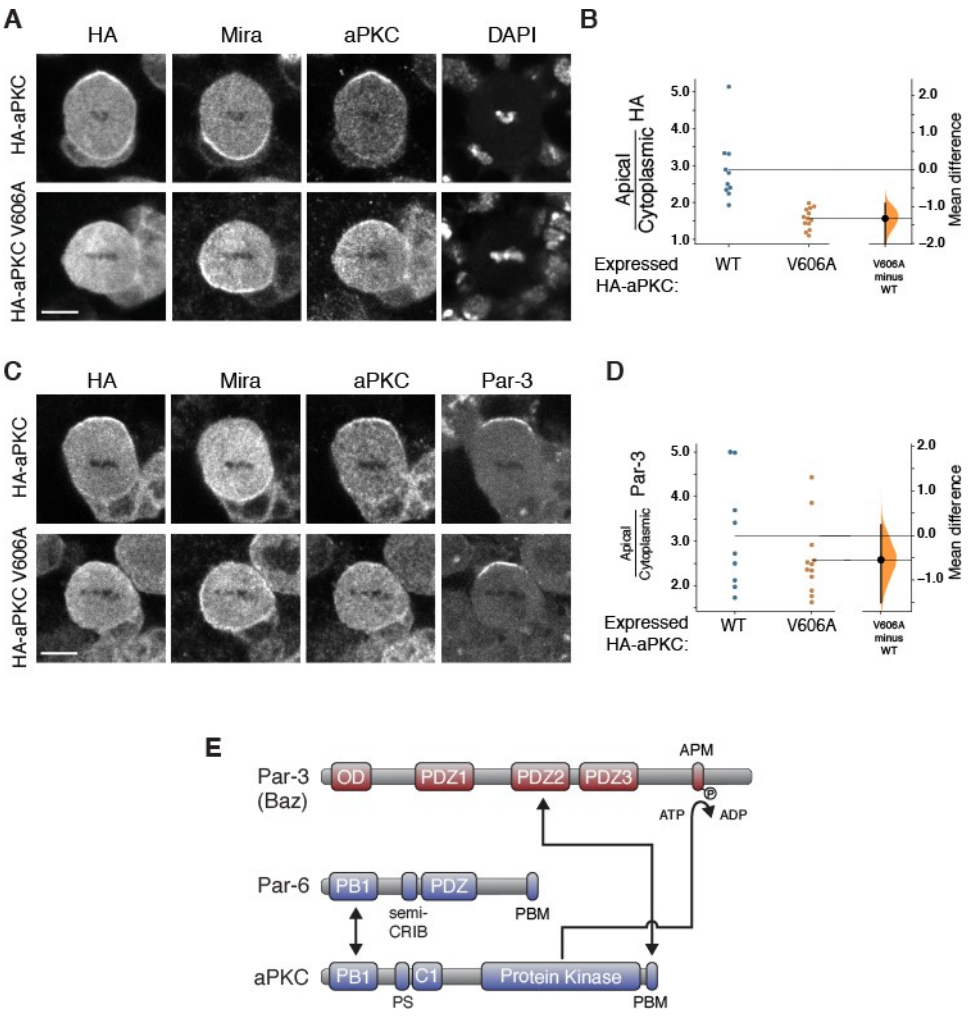


**Figure 8: aPKC polarization requires its PDZ Binding Motif.**
(A) Protein localization in metaphase neuroblasts expressing WT or V606A aPKC. The localization of HA-tagged WT or V606A aPKC, expressed using Worniu-GAL4/UAS, is shown with the basal marker Miranda, total aPKC (transgenically expressed and endogenous) using an anti-aPKC antibody, and DNA (DAPI). Scale bar is 5 µm. A similar analysis in *aPKC* mutant neuroblasts is shown in Figure S2. (B) Gardner-Altman estimation plot of effect of the aPKC V606A mutation on aPKC cortical localization. Ratios of apical cortical to cytoplasmic anti-HA signal intensities are shown for individual metaphase neuroblasts expressing either HA-WT or HA-V606A aPKC. Statistics: Bootstrap 95% confidence interval (bar in "V606A minus WT" column). (C) Localization of Par-3 in metaphase neuroblasts expressing WT or V606A aPKC, as in panel d. (D) Gardner-Altman estimation plot of effect of the aPKC V606A mutation on Par-3 cortical localization. Apical cortical to cytoplasmic signal intensities of anti-Par-3 signals are shown for individual metaphase neuroblasts expressing either HA-WT or HA-V606A aPKC. Statistics: Statistics: Bootstrap 95% confidence interval (bar in "V606A minus WT" column). (E) Par-3 interactions with Par-6/aPKC analyzed in this study. The Par-3 PDZ2–aPKC PBM interaction forms a persistent connection while the aPKC kinase domain interacts transiently with the Par-3 APM when ATP is present.

neuroblasts lacking endogenous aPKC (Figure S2). We conclude that the Par-3 PDZ2-aPKC PBM interaction is required for cortical recruitment and polarization of aPKC in neuroblasts.

We have examined the interaction of Par-3 with the full-length Par complex and found that Par-3 PDZ2 and a previously unrecognized PBM at the COOH-terminus of aPKC are required for complex assembly (Figure 8E). The Par-3 phosphorylation site (APM) can also form a persistent interaction with the aPKC kinase domain, but only if phosphorylation is not allowed to occur due to the absence of ATP. Unlike the APM–kinase domain interaction, the Par-3 PDZ2 interaction with the aPKC PBM is not influenced by the presence of ATP, suggesting that additional mechanisms besides APM phosphorylation must exist to dissociate Par-3 from the Par complex, an important component of current polarity models (Morais-de-Sá et al., 2010; Rodriguez et al., 2017; Wang et al., 2017).

The identification of Par-3 PDZ2 domain as a key factor in recruiting the Par complex to the cortex during animal cell polarization is consistent with previous work demonstrating that while Par-3 PDZ1 and 3 are dispensable in *C. elegans,* PDZ2 is required for cortical recruitment of Par-6 and aPKC (Li et al., 2010a). It is also consistent with work in both *C. elegans* and *Drosophila* showing that the interaction of Par-6 with Par-3 is not required (Li et al., 2010b; Renschler et al., 2018). In *Drosophila*, the role of PDZ2 is less clear but is known to be required for downstream effects on epithelial structure (McKinley et al., 2012). We suggest that the Par-3 PDZ2 – aPKC PBM interaction represents an important physical connection for animal cell polarity and that

the reconstitution approach used to identify this interaction will likely be useful for understanding how other regulatory molecules, such as Cdc42, control polarity.

## STAR METHODS

### Lead contact and materials availability

All request for reagents should be directed to Lead Contact, Ken Prehoda. (prehoda@uoregon.edu)

### Experimental model and subject details

*Animals: Drosophila*

A mix of male and female larvae were used for all *in vivo* experiments. The strains used in this study were: ;Worniu-Gal4 (BDSC_56553), ; FRT-G13, aPKC$^{K06403}$/CyO (Gift from C.Q. Doe), and elav-Gal4, UAS-mCD8:GFP, hs:flp; FRT-G13, tubPGal80 (BDSC_5145).

In addition, two fly lines were created for this study, 3xHA-aPKC 1-606 and 3xHA-aPKC V606A. These were made using Phi-C31 integration. In brief, the coding region of aPKC wild-type (1-606) (A1Z9X0) or aPKC PBM mutant (V606A) were cloned into a pUAST vector (GenBank: EF362409.1) modified to contain an N-terminal 3xHA tag. Vector was injected into attP2 containing flies in a *y, w* background (BDSC_8622) and integrated using PhiC31 (BestGene, Inc.). F1 generation progeny were backcrossesd to *y, w* adults and F2 progeny were screened for the presence of red eyes.

**Method details**

*Expression and Purification of Par-complex*

Plasmids (pCMV) containing the coding sequences for aPKC and His-Par-6 including the mutants aPKCΔPBM or His-Par-6ΔPBM were co-transfected into HEK293-F suspension cells using 293fectin (Thermo Fisher) and grown in shaker flasks for 60h at 37°C. Cells were collected by centrifugation at 1000 RPM x 3 minutes, resuspended in lysis buffer (50 mM $NH_3PO_4$, 300 mM NaCl, 10 mM Imidazole, pH 8.0), and lysed by probe sonication at 50% amplitude, 0.3s/0.7s pulse on/off, 3 x 1 minute. After clearing cellular debris by centrifugation at 15k RPM x 20 minutes, protein was gently mixed with 4mL HisPur Cobalt (ThermoFisher) resin for 45 minutes. Resin was washed twice with 20mL lysis buffer supplemented with 5 mM $MgCl_2$ and 100 µM ATP followed by a final wash with 20mL nickel lysis buffer. Protein was eluted with elution buffer (50 mM $NH_3PO_4$, 300 mM NaCl, 300 mM Imidazole, pH 8.0) by gravity and buffer exchanged with 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, and 100 µM ATP using a PD10 desalting column (GE Healthcare). Finally, protein was purified by Source Q anion exchange chromatography with a salt gradient from 100 mM to 550 mM. Fractions from the elution peak containing aPKC phosphorylated at the activation loop and turn motif, as verified by reactivity with phosphospecific antibodies (Rabbit $\alpha\alpha$-PKCζ p-410 Santa Cruz Biotech, sc-12894-R; Rabbit $\alpha\alpha$-PKC p560 Abcam ab62372), were pooled concentrated to 2.5mL, and buffer shifted to 20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 100 µM ATP, and 1 mM DTT, followed by concentration to ~400 µL. Protein was aliquoted, flash frozen, and stored at -80°C until use. Par-complex was quantified by western blot analysis using a standard curve

generated with a sample of known concentration using an anti-aPKC antibody (Mouse $\alpha\alpha$-PKCζ H-1 Santa Cruz Biotech sc-17781).

*Expression and Purification of Par-3.*

Par-3 PDZ1 – APM (aa 309 – 991) was cloned (Gibson Cloning) into the pMal expression vector allowing for an $NH_2$-terminal MBP tag as well as a COOH-terminal $His_6$ tag to allow for dual affinity purification. Full length Par-3 (aa 1 - 1464) and all full length Par-3 domain deletion mutants were cloned into the pMal expression vector allowing for an $NH_2$-terminal MBP tag. Plasmids were transformed into E. *coli* BL21 (DE3) cells, plated on LB + AMP and allowed to grow for 18h at 37 C. Single colonies were picked to inoculate 100 mL of LB + AMP starter culture and grown for ~4h. Starter culture was used to inoculate 2L LB + AMP and cultures were grown to an $OD_{600}$ of 0.8 – 1, followed by a 3h induction with 500 μM IPTG. Cell pellets were collected at 5000 RPM x 20 minutes and resuspended in lysis buffer (50 mM $NH_3PO_4$, 300 mM NaCl, 10 mM Imidazole, pH 8.0). Cell extracts were thawed under running water and lysed by probe sonication at 70% amplitude, 0.3s/0.7s pulse on/off, 3 x 1 minute. Cellular debris was cleared at 15,000 RPM x 20 minutes and supernatant was added to 5mL HisPur cobalt resin and incubated for 30 minutes at 4 C. Resin was washed 3 times with lysis buffer followed by elution with nickel elution buffer (50 mM $NH_3PO_4$, 300 mM NaCl, 300 mM Imidazole, pH 8.0). Fractions containing protein were pooled and concentrated to 2.5mL. Proteins were buffer shifted over PD10 desalting columns (GE Healthcare) to 20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM DTT followed by flash freezing and storage at -80°C. Protein was quantified by Bradford and purity was assessed by SDS-Page. All

other Par-3 constructs were cloned into pMal, pGex, or pET expression vectors allowing for a single $NH_2$-terminal MBP, GST, or His tag. Proteins were expressed in transformed BL21 (DE3) competent cells as above. MBP-protein cell extracts were resuspended in MBP lysis buffer (20mM Tris, pH 7.5, 200mM NaCl, 1mM EDTA, 1mM DTT). GST-protein cell extracts were resuspended in GST lysis buffer (1x PBS, pH 7.4, 1mM DTT). His-protein cell extracts were resuspended in nickel lysis buffer (50 mM $NH_3PO_4$, 300 mM NaCl, 10 mM Imidazole, pH 8.0). Cells were lysed and cleared as above. For proteins used as bait in pull down assays, cleared lysate was aliquoted, flash frozen, and stored at -80 C. Purified proteins were prepared by incubating cleared lysates with 5mL of amylose, glutathione, or HisPur cobalt resin for 30 minutes at 4 C. Resin was washed 3x with either GST, MBP, or nickel lysis buffer. Proteins were then eluted with MBP, GST, or Nickel lysis buffer supplemented with 10 mM maltose, 10mM glutathione, or 300mM Imidizole, respectively. Fractions containing protein were pooled and concentrated to 2.5mL with Vivaspin 20 centrifugal concentrators. Proteins were buffer shifted to 20mM HEPES, pH 7.5, 100mM NaCl, 1mM DTT. Finally, proteins were concentrated to 500 µL, aliquoted, flash frozen, and stored at -80 C.

*Affinity chromatography interaction assay*

Amylose or glutathione resin was loaded with bacterial lysate (or his purification elutions in the case of Par-3 PDZ1-APM or Par-3 PDZ1-PDZ3 as these proteins contain COOH-terminal his tags) containing MBP- or GST-fusion protein for 30 minutes at 4° C and then washed with wash buffer three times (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% Tween 20, and 1 mM DTT). Par-complex was then added to a

concentration of 0.5 μM and incubated for 10 minutes at room temperature. In the case where ATP is present, ATP was used at a final concentration of 200 μM in all buffers throughout the pull down experiment and and binding reactions were carried out for 30 minutes at room temperature. Finally, beads were washed two times briefly to remove unbound Par-complex and beads were resuspended in loading dye. Samples were analyzed by SDS-Page and stained by Coomassie as well as Western Blot using $\alpha\alpha$-aPKC (Mouse $\alpha\alpha$-PKCζ H-1 Santa Cruz Biotech sc-17781) and rat $\alpha\alpha$-Par-6.

*Supernatant depletion interaction assay*

Amylose resin was loaded with bacterial lysate containing MBP – Par-3 PDZ1 – APM for 30 minutes at 4° C and then washed with wash buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 200 μM ATP, 0.5% Tween 20, and 1 mM DTT). 2-fold serial dilutions of beads were prepared from 20 μL to 0.625 μL in a total volume of 200 μL. Par-complex was added to a final concentration of 40 nM diluted in wash buffer. After incubation for 30 minutes, beads were collected by centrifugation and an aliquot of supernatant was diluted in loading dye for western blot analysis using $\alpha\alpha$-aPKC (Mouse $\alpha\alpha$-PKCζ H-1 Santa Cruz Biotech sc-17781) and rat $\alpha\alpha$-Par-6. The concentration of protein loaded on the beads was verified by SDS-Page using a standard curve generated with known concentrations of BSA.

*Drosophila Neuroblast Immunofluoresence*

Flies were allowed to lay in vials for 24 hours at room temperature, after which the flies were removed, and the vial was moved to 30˚C. During wandering third instar

larval stage (5-6 days later), larvae were dissected within 20 minutes into Schneider's Insect Medium (Sigma, S0146). Brains were fixed using 4% PFA in PBS for 20 minutes followed by 3 washes in PBST (1xPBS + 0.3% Triton-X 100, Sigma-Aldrich). Note that during all fixing, blocking, and washing steps, brains were kept moving on a nutator. At this stage brains could be kept for up to 3 days nutating at 4˚C in PBST or washed an additional 20 minutes in PBST before blocking and staining. Brains were blocked for 30 minutes in PBSBT (PBST + 1% BSA, Fisher Scientific). Incubation in primary antibodies occurred overnight at 4˚C. Primary antibodies: Rat $\alpha\alpha$-Mira (1:500; Abcam, ab197788), Mouse $\alpha\alpha$-PKCζH-1(1:1,000; Santa Cruz Biotech sc-17781), Rabbit $\alpha\alpha$-PKCζ C-20 (1:1,000; Santa Cruz Biotech sc-216), Rabbit $\alpha\alpha$-HA C29F4(1:1,000; Cell Signaling Technologies, 3724), Mouse $\alpha\alpha$-HA (1:500; Covance, MMS-101P), and Guinea Pig $\alpha\alpha$-Baz(1:2,000; gift from C.Doe). Brains were then washed quickly followed by 3x15 minutes washes in PBSBT. Incubation in secondary antibodies occurred at room temperature protected from light for 2 hours followed by a quick wash and 3x15 minutes washes in PBST. Secondary antibodies used were from Jackson Immunoresearch Laboratories, Inc.: Dk $\alpha\alpha$-Rt Cy3 (712-165-153; 1:500), Dk $\alpha\alpha$-Rb 647 (711-605-152; 1:500), Dk $\alpha\alpha$-Ms 488 (715-545-151), Dk $\alpha\alpha$-Gp 405 (706-475-148; 1:500). Brains were stored in SlowFade Diamond with DAPI (Invitrogen, S36964) for at least 24 hours before imaging. Brains were imaged using a Leica TCS SPE upright confocal microscope using an ACS APO 40x 1.15 NA Oil CS objective.

*Drosophila Neuroblast MARCM Clones*

To generate *Drosophila* neuroblast MARCM clones, FRT-G13, *aPKC*$^{K06403}$/CyO Virgin flies were crossed to ;;3xHA-aPKC V606A male flies. The resulting non-Cyo male progeny were crossed to elav-Gal4, UAS-mCD8:GFP, hs:flp; FRT-G13, tubPGal80 Virgins and allowed to lay for 24 hours at room temperature. The vials were then allowed to stay at room temperature for an additional 24 hours at which time they were heat-shocked @37˚C for 90 minutes. This was followed by a possible second 90 minute heat-shock within 18 hours. Vials were raised at 18˚C or room temperature until wandering third instar stage when they were dissected and stained as described above with the following antibodies: Primary antibodies: Rat $\alpha\alpha$-Mira (1:500; Abcam, ab197788), Rabbit $\alpha\alpha$-HA C29F4(1:1,000; Cell Signaling Technologies, 3724) or Mouse $\alpha\alpha$-HA (1:500; Covance, MMS-101P), and Chicken $\alpha\alpha$-GFP (1:500; Abcam, ab13970). Secondary antibodies: Dk $\alpha\alpha$-Rt Cy3 (712-165-153; 1:500), Dk $\alpha\alpha$-Rb 647 (711-605-152; 1:500) or Dk $\alpha\alpha$-Ms 647 (715-605-151; 1:500), and Dk $\alpha\alpha$-Ck 488 (703-545-155). Brains were imaged using a Leica TCS SPE upright confocal microscope using an ACS APO 40x 1.15 NA Oil CS objective or an Olympus Fluoview FV1000 upright confocal microscope using a PlanApo N 60x 1.42 NA Oil objective.

**Quantification and statistical analysis**

*Quantification of equilibrium dissociation constants*

The equilibrium dissociation constant for Par-3 PDZ1-APM binding to Par-6/aPKC was calculated by measuring both Par-6 and aPKC western signals from the supernatant of solutions containing increasing concentrations of amylose resin-bound MBP-Par-3 PDZ1-APM and fitting to the following equation:

$$f_b = \text{free} + (\text{bound} - \text{free})*[\text{Par-3 PDZ1-APM}]/([\text{Par-3 PDZ1-APM}] + K_d)$$

"$f_b$" was calculated from the experimentally measured Par-6 and aPKC western signals using the equation $1 - W_x/W_0$ where $W_x$ is the western signal at Par-3 concentration x and $W_0$ is the western signal in the absence of Par-3

"free" and "bound" are the fraction of Par-6/aPKC bound when saturated and in the absence of Par-3, respectively. These parameters were allowed to float

$K_d$ is the equilibrium dissociation constant.

Fitting and calculation of 95% confidence intervals were done using the LMFIT python package.

*Drosophila Neuroblast Quantification*

All images were analyzed using Fiji. For quantification of apical cortical to cytoplasmic signal intensity ratios, corresponding signals were measured from an intensity profile averaged from 10 pixels across the apical portion of the cell parallel with the polarity axis in a central optical section. The apical value was taken as the highest peak data point corresponding with the apical domain of the cell, the cytoplasmic value was an average of 20 data points that were a distance of 10 points away from the apical value.

**Bridge to Chapter IV:**

Chapter III, introduced the messy field of determining how Par3 and the Par complex interact. In the literature, there were at least 5 interactions described, but which

ones were required for polarity was unknown. To address this, we used a combination of *in vitro* biochemical and *in vivo Drosophila* genetics to show that none of the previously identified interactions were required. Instead a novel interaction between the previously uncharacterized aPKC c-terminal PBM and the PDZ2 of Par3 is required for polarization of aPKC in *Drosophila* neuroblasts.

In Chapter IV, I summarize what is contained in the previous three chapters. I then discuss what the implications of the discoveries outlined in Chapter II and III are for understanding how these systems function and future work in cell polarity.

# CHAPTER IV

# DISCUSSION AND CLOSING THOUGHTS

This chapter contains unpublished material written by K.A.J.

## SUMMARY

Chapter I introduced the role of the evolutionarily conserved Par complex (the kinase aPKC and its adaptor protein Par6) in the regulation of animal polarity across a variety of cell types and organisms. This complex is known to colocalize with and be regulated by the PDZ scaffold protein Par3 and the small GTPase Cdc42. Across all systems studied, one or both of these regulators is required for recruitment of the Par complex to the apical (or anterior) domain of polarized cells (Goldstein and Macara, 2007). The literature suggests that these interactions may be sufficient for membrane recruitment of the Par complex (Lang and Munro, 2017). However there are multiple examples of depolarized Par complex in different mutational backgrounds that have not been taken into account with this model (Atwood and Prehoda, 2009; Chabu and Doe, 2008; Chabu and Doe, 2009; Lee et al., 2006a; Lee et al., 2006b; Oon and Prehoda, 2019; Wang et al., 2009a). As we want to know the direct, specific interactions that get the Par complex to the cortex as a key to understanding how the Par complex is polarized, this suggests that further study is needed.

Chapter II reviewed recent research from *C. elegans* and *Drosophila* that has shown that kinase inactive mutants of aPKC can display depolarized localization.

However it is not fully clear whether these mutants rely on the upstream regulators Par3 and Cdc42 for their localization (Hannaford et al., 2019; Rodriguez et al., 2017). This chapter sought to clarify the mechanism of localization of these kinase inactive mutants. Using a genetics approach, we found that kinase inactive mutants of aPKC show depolarized localization to the entire cortex, but this localization is not due to lack of kinase activity itself or the regulators Par3 and Cdc42. Through a structure function analysis of aPKC, we showed that the C1 domain of aPKC is sufficient to localize to the entire cortex and can serve as a general lipid binding motif by binding directly to phospholipids. This domain is required for cortical targeting and polarization of aPKC. Additionally we believe that the C1 domain is regulated through intramolecular interactions involving both the PB1 and kinase domains. Regulation of the C1 appears to be important as all aPKC constructs that showed depolarized localization were constitutive and lacked cell cycle polarity regulation. This work adds a third, direct interaction to the polarization model for neuroblasts.

Chapter III reviewed the interactions that have been discovered to occur between Par3 and aPKC. These interactions are varied and map to both members of the Par complex (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Renschler et al., 2018; Soriano et al., 2016; Wodarz et al., 2000). This chapter sought to determine which interactions were actually required for interaction between Par3 and the Par complex. Using a biochemical approach with purified components, we found that none of the previously described interactions was required for aPKC and Par3 to interact. Instead a new interaction with a novel PDZ Binding Motif (PBM) at the C-terminus of aPKC with

the PDZ2 domain of Par3 was required. We also showed that this interaction is required for aPKC polarization *in vivo*, as aPKC PBM mutants localized to the cytoplasm in *Drosophila* larval neuroblasts.

## DISCUSSION

### Chapter II:

### Implications of C1 lipid interactions

aPKC is considered atypical for two reasons: it contains an N-terminal PB1 domain that is not shared by any other class of PKC (novel or conventional) and its irregular C1 domain. The majority of other PKCs contain more than one C1 domain and often a secondary C2 domain that is activated by calcium signaling. It is through the activation of the C2 domains and the C1 domain by binding to diacylglycerol (DAG) that novel and conventional PKCs are able to localize to membranes, a key step in their maturation and activity (Rosse et al., 2010). However aPKC contains mutations of the key residues that allow for DAG binding, changing them from hydrophobic residues to charged arginines (Pu et al., 2006). This renders atypical C1s insensitive to DAG in two ways. First DAG localizes in the membrane just below the headgroups of the surrounding phospholipids so typical C1s must partially insert into the membrane requiring hydrophobic residues on the surface that interacts with DAG. Secondly C1 domains contain two loops on their binding face that DAG can insert between. In aPKC C1, these loops are in a position due to the arginines that narrows the cleft between them, becoming too small for DAG to bind (Pu et al., 2006). However it has been hypothesized that smaller lipids, such as ceramide may be able to interact with the cleft of atypical C1s (van

Blitterswijk, 1998). This idea is especially interesting as lipids have long been known to be activators of atypical PKCs *in vitro* (Suzuki et al., 2003). Perhaps this is part of the activation mechanism for aPKCs.

This work shows that lipid binding ability of aPKC C1 is still maintained similar to conventional and novel PKCs but just relies on different lipids. Although we showed that the C1 can bind to a variety of phospholipids, it may still show some preference in cells. Specifically many cortical proteins are known to rely on phosphatidylinositides (PIPs) which make up only ~1% of the plasma membrane for their localization. These PIPs can have a varied number of phosphates attached which can change the specificity of the proteins that bind to them. Additionally PIPs have been shown to be polarized in different cell types with implications for cell polarity (Rodriguez-Boulan and Macara, 2014). Since aPKC has also been shown to be activated by $PIP_3$ specifically *in vitro*, this gives another possibility for regulation of the C1 domain *in vivo* (Ivey et al., 2014).

**Is the cortical targeting role of the C1 domain conserved in other contexts?**

As aPKC kinase inactive mutants in *C. elegans* have been shown recently to localize to the entire cortex, could this new interaction also explain something about this localization in other contexts.

*C. elegans* start with anterior Pars cortical while *Drosophila* neuroblasts start with aPKC/Par6 cytoplasmic and Par3 punctate on cortex (Oon and Prehoda, 2019; Reich et al., 2019). Comparing the work of Rodriguez et al. to Chapter II, this would suggest another possible difference, since the kinase inactive aPKC ts and CRT90 treated embryos did not rely on Par3 but did rely on Cdc42 for their localization. Although this

may actually be more nuanced as aPKC was able to remain on the cortex (expanded from anterior) during establishment but was mostly cytoplasmic during maintenance (Rodriguez et al., 2017). Does the C1 of *C. elegans* aPKC have a role in initial targeting of the protein? Are other interactions required to maintain it on the cortex? This could be reminiscent of *Drosophila* photoreceptors that rely on Cdc42 and Par3 for localization of the Par complex, but they also rely on the apical protein Crb to stabilize enough protein in the apical domain (Nunes de Alemeida, 2019).

Work in MDCK cells has previously shown that expression of the N-terminal regulatory region of PKC zeta shows cortical localization, suggesting that this cortical targeting ability of the C1 domain may be conserved in other organisms (Seidl et al., 2012).

This also gives a new paradigm to view other instances of depolarized aPKC in the literature (*Dap160*, latrunculin treatment, *AuroraA*, *PP2A*) (Atwood and Prehoda, 2009; Chabu and Doe, 2008; Chabu and Doe, 2009; Lee et al., 2006a; Lee et al., 2006b; Oon and Prehoda, 2019; Wang et al., 2009a). Can these depolarized aPKC seen in these mutational contexts be explained by the current model or do they rely on misregulation of the C1 domain?

**What is the mechanism of C1 regulation?**

As was showed in Chapter II, the C1 domain is inhibited intramolecularly by the PB1 and Kinase domains of aPKC. Additionally in their deuterium exchange assay, Lopez-Garcia et al. showed only two areas on the kinase domain that were protected that could be traced to the pseudosubstrate and the C1 domain (Lopez-Garcia et al., 2011).

Since the PB1 domain appears to serve in the C1's inhibition, it is interesting to think that this may be direct.

What is the actual mechanism of the C1 activation? Is it exposed upon Par6 binding or does it only become exposed once Par6/Cdc42 and Par3 are bound to the complex providing a necessary stabilization interaction at the membrane? This could explain how the initial targeting of the complex is only within the apical half of the cell in *Drosophila* neuroblasts upon mitotic entry (Oon and Prehoda, 2019).

Another interesting thought is how the Par complex becomes located to the cytoplasm once mitosis is over in neuroblasts. If the C1 domain is exposed when in the apical domain, how does it become inhibited again?

**Does the pseudosubstrate of aPKC have a stabilizing role in polarity?**

Mutation of the pseudosubstrate did not appear to be required for polarization, although the amount of this aPKC mutant at the apical domain was reduced compared to FL aPKC. This would suggest that the pseudosubstrate may have a stabilizing effect once aPKC is at the cortex.

The pseudosubstrate has been implicated in *in vitro* studies that show activation of aPKC by lipid addition (Ivey et al., 2014). Perhaps its role is to help interact with lipids along with the C1 domain. Additional research in this area will prove interesting.

**Chapter III:**

**What is the consequence of the newly discovered Par3 interaction with aPKC?**

Chapter III narrowed the field of potential aPKC/Par3 interactions down to one that was previously undescribed the PBM of aPKC with the PDZ2 of Par3. What are those other interactions doing? Are they required for polarity in a specific context?

It is possible that the report that narrowed down aPKC binding to the Par3 PDZ2 and PDZ3 domains could have been due to this new interaction as all of the parts: aPKC PBM and Par3 PDZ2 were present (Wodarz et al., 2000). Also we know that the transient interaction between the kinase domain and the APM of Par3 are required for proper epithelial cell junctions, but this interaction does not contribute to a stable complex and therefore would not be relevant for aPKC polarization (Holly and Prehoda, 2019). The Par6 interactions with Par3 are particularly interesting in light of a recent report, suggesting that the Par complex may actually exist in higher order structures (Dickinson et al., 2017). Perhaps these structures could be formed as a stabilizing interaction once the Par complex is already at the apical cortex but is not required for the initial recruitment. One consequence of creating such a structure could be to maintain the apical localization of the Par complex by limiting the diffusion that occurs at the interface with the rest of the cytoplasm.

## CONCLUDING REMARKS

This goal of this work was to determine what the specific, direct interactions are that are required for cortical targeting and polarization of the Par complex. This work introduced a third, direct interaction that aPKC must have to polarize in *Drosophila* neuroblasts. It also clarified the interactions required for interactions between aPKC and Par3 by describing a novel interaction between the aPKC PBM and the PDZ2 of Par3.

This interaction appears to be the only required interaction and is required in *Drosophila* neuroblasts for aPKC polarization. It will be interesting to see what the implications of these new interactions are especially in terms of conservation in other contexts and systems.

# APPENDIX

## APPENDIX A: SUPPLEMENTAL MATERIAL FOR CHAPTER II

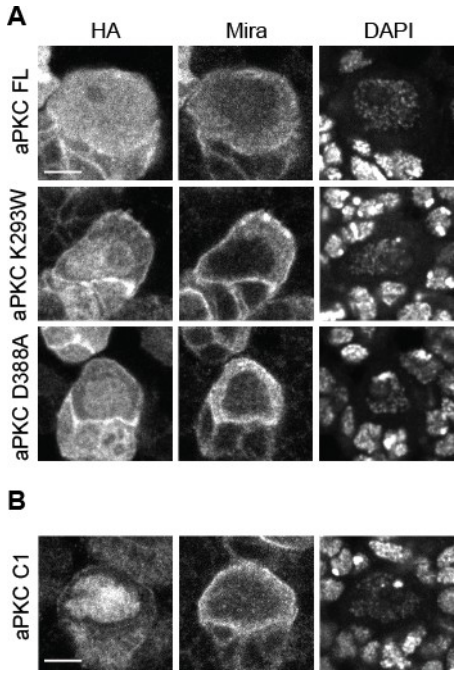


**Figure S1: aPKC constructs that are cortical in metaphase also display interphase cortical and nuclear localization.** (A) HA, Mira, and DAPI staining of HA-aPKC FL, K293W, and aPKC D388A expressed in interphase larval neuroblasts with 1407Gal4/UAS. (B) HA, Mira, and DAPI staining of HA-aPKC C1 domain expressed in interphase larval neuroblasts with 1407Gal4/UAS.
Scale Bars = 5μm.

# APPENDIX

## APPENDIX B: SUPPLEMENTAL MATERIALS FOR CHAPTER III

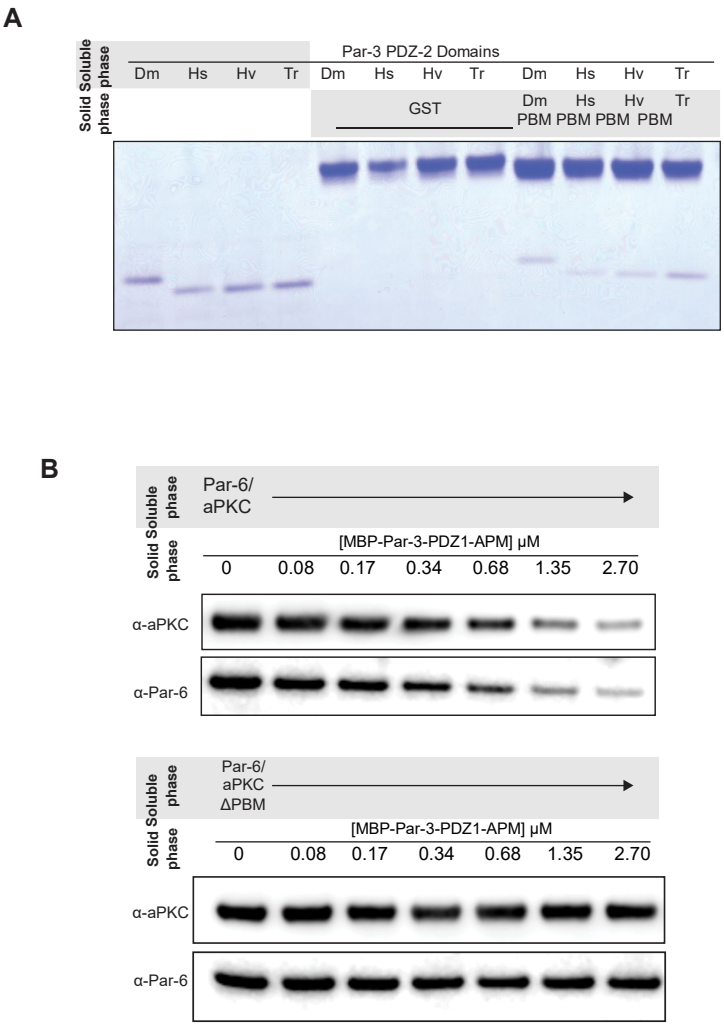


**Figure S1: Binding of Par-3 PDZ2 and aPKC PBM orthologues from diverse metazoan organisms and quantitative analysis by supernatant depletion. Related to Figure 3.** (A) Solid phase (glutathione resin) bound Glutathione-S-Transferase (GST) fused aPKC PBMs with Par-3 PDZ2 domains from the indicated organisms (Dm = *Drosophila melanogaster*; Hs = *Homo sapiens*; Hv = *Hydra vulgaris*; Tr = *Trichoplax sp H2*). Shaded region indicates fraction applied to gel (soluble phase or solid phase components after mixing with soluble phase components and washing). (B) Equilibrium binding of Par-3 PDZ1-APM to Par-6/aPKC (top panel) or Par-6/aPKCΔPBM (bottom panel). Westerns show aPKC and Par-6 remaining in the supernatant after incubation with solid phase (amylose resin) bound with the indicated concentration of Maltose Binding Protein (MBP) fused Par-3 PDZ1-APM. Shaded region indicates fraction applied to gel after mixing and incubation.

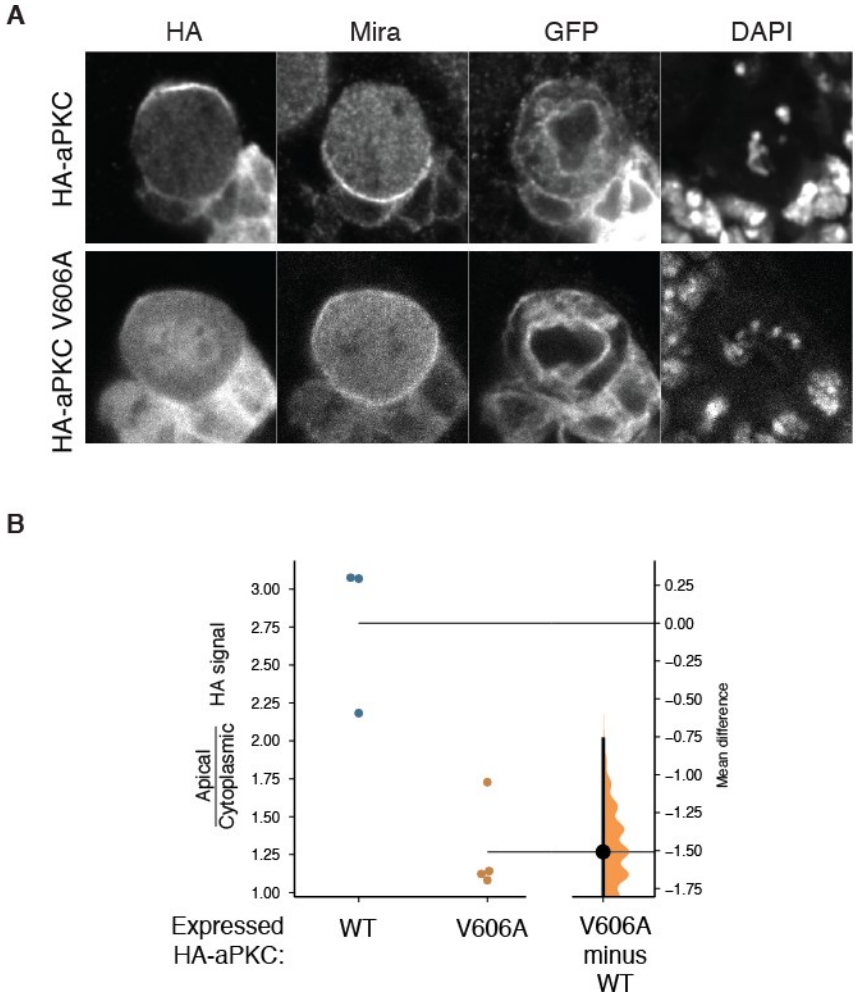


**Figure S2: Recruitment of aPKC to the apical cortex of metaphase *Drosophila* neuroblasts depends on its PDZ Binding Motif. Related to Figure 4.** (A) Protein localization in metaphase *aPKC*$^{K06403}$ neuroblasts expressing WT or V606A aPKC. The localization of HA-tagged WT or V606A aPKC, expressed using Worniu-Gal4/UAS, is shown with the basal marker Miranda, GFP-mCD8 (marks *aPKC*$^{K06403}$ neuroblasts), and DNA (DAPI). (B) Gardner-Altman estimation plot of effect of the aPKC V606A mutation on aPKC cortical localization. Apical cortical to cytoplasmic signal anti-HA intensities are shown for individual *aPKC*$^{K06403}$ metaphase neuroblasts expressing either HA-WT or HA-V606A aPKC. Statistics: Bootstrap 95% confidence interval (bar in "V606A minus WT" column).

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