# Functional analysis of the Bazooka protein in the establishment of cell polarity in *Drosophila melanogaster*

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# 1. Zusammenfassung

Für Komponenten des sogenannten PAR/aPKC- (partitioning-defective / atypische Proteinkinase C) Komplexes wurde nachgewiesen, dass sie eine Schlüsselrolle in der Entstehung und Erhaltung der Zellpolarität in unterschiedlichen Zelltypen spielen. Die grundlegenden Mechanismen scheinen hierbei in der Evolution zwischen Wurm und Mensch stark konserviert zu sein. Forschung an der Fruchtfliege *Drosophila melanogaster* hat gezeigt, dass Bazooka als Kernkomponente des PAR/aPKC Komplexes an der Spitze einer komplexen Hierachie steht, die die Zellpolarität reguliert. Nicht nur für die Etablierung der Zellpolarität in epithelialen Zellen, sondern auch für die asymmetrische Zellteilung der neuralen Stammzellen (Neuroblasten) und für die Determinierung der Schicksale der beiden Tochterzellen ist die asymmetrische Lokaliserung von Bazooka essentiell. Trotzdem ist immer noch nicht geklärt, wie genau Bazooka selbst an die Membran lokalisiert wird und wie diese Rekrutierung während der Etablierung der Zellpolarität reguliert wird.

In der vorliegenden Studie wurde eine systematische Strukturanalyse des Bazooka-Proteins vorgenommen, indem Fusionsproteine aus Bazooka-Deletionskonstrukten und dem grünen fluoreszierenden Protein (GFP) in transgenen Fliegen und in der Zellkultur exprimiert wurden. Dabei wurde festgestellt, dass die C-terminale Region von Bazooka ein neues Lipid-Bindemotiv enthält und essentiell für die Membranlokalisierung des Proteins ist.

Des weiteren wurde die Rolle von zwei Phosphorylierungen näher untersucht: Zum einen die Phosphorylierung und Dephosphorylierung des konservierten Serinrestes 1085 durch die Kinase PAR-1 und die Phosphatase PP2A, wodurch die apikal-basale Polarität in Neuroblasten kontrolliert wird. Dies geschieht durch die Regulierung einer Bindestelle für die Adaptorproteine 14-3-3ε und Leonardo. Defekte in dieser Signalkaskade führen in einem hohen Anteil embryonaler Neuroblasten zu einer Umkehr der apikal-basalen Polarität.

Zweitens wurde die Interaktion zwischen Bazooka und aPKC, welches Bazooka an dem konservierten Serinrest 980 phosphoryliert, genauer charakterisiert. Hierbei konnte gezeigt werden, dass die Überexpression einer nicht phosphorylierbaren Variante von Bazooka zu einem drastischen dominant-negativen Phänotyp führt, der mit einem Verlust der Zellpolarität und embryonaler Letalität verbunden ist.

# 2. Summary

Components of the PAR/aPKC (partitioning-defective / atypical protein kinase C) complex have been found to play a key role in the establishment and maintenance of cell polarity in various cell types. The underlying mechanisms are highly conserved throughout evolution, from worm to mammals. Research in the fruit fly *Drosophila melanogaster* revealed that Bazooka as the core component of the PAR/aPKC complex acts on top of a hierarchy in the regulation of cell polarity. Not only the establishment of epithelial cell polarity, but also the asymmetric cell division of the neural stem cell (neuroblast, NB) and the determination of the distinct cell fates of the two daughter cells are dependent on asymmetric localization of Bazooka. However, it is not yet fully elucidated how exactly Bazooka itself is localized to the apical membrane domain and how its targeting is regulated during the establishment and maintenance of cell polarity.

In this study, a systematic structural analysis of the Bazooka protein was performed, using deletion constructs tagged with green fluorescent protein (GFP) in transgenic flies and in cell culture experiments in order to clarify the role of the distinct domains of the protein. We found that the C-terminal region of Bazooka, contains a new lipid binding motif and is crucial for membrane association of the protein.

Furthermore, the role of two different phosphorylation events of Bazooka were elucidated: First, (de)phosphorylation at the conserved serine residue 1085 by the kinase PAR-1 and the phosphatase PP2A controls apical-basal polarity in dividing embryonic NBs by regulating a binding site for the adaptor proteins 14-3-3ε and Leonardo. Defects in this pathway lead to frequent reversal of apical-basal polarity in embryonic NBs.

Second, the interaction of Bazooka with aPKC, which phosphorylates Bazooka at the conserved serine residue 980, was investigated in more detail. Overexpression of a non-phosphorylatable version of Baz leads to a drastic dominant negative phenotype with a total loss of cell polarity and embryonic lethality.

# 3. Introduction

## 3.1. Cell polarity

Cell polarity is one of the key features of multicellular organisms and is the prerequisite for various complex functions including the establishment of epithelial barriers, directed growth and movement and the three dimensional development of the nervous system.

After more than one century of intensive research we are far from understanding the interactions of genes, proteins and regulatory RNAs involved in the regulation of cell polarity, and many pieces of this puzzle remain to be identified. Nevertheless, some common principles and key players of polarity have been revealed and investigated. Interestingly, most of them are well conserved throughout evolution and have a general function in different polarized cell types.

The approach of developmental biology and the work on model organisms like *Drosophila melanogaster* provides versatile tools not only for the understanding of fundamental mechanisms of life and diseases but also for the development of specific drugs and therapies. In contrast to mammalian cell culture systems, the fruit fly *Drosophila* offers not only the opportunity of a real “in vivo” approach to test all mechanisms, mutations, candidates etc. for their implications on the entire organism. It also allows to investigate them in different cell types, tissues and developmental stages and thereby to compare directly the underlying mechanisms.

In *Drosophila*, at least five different polarized cell types are easily accessible for in vivo research:

1. The oocyte, which is surrounded by the follicle epithelium exhibits an anterior (facing the nurse cells) – posterior (facing the next egg chamber) - polarity, which is reflected not only by the specific localization of proteins but also by the directed, microtubule based transport and localization of mRNAs.

2. The ectodermal epithelium surrounds the developing embryo, secreting a protective cuticle. It also forms part of the intestinal system, the tracheae and the salivary glands (see also 2.2).

3. The mesodermal follicle cell epithelium. Similar to the ectodermal cells of the epidermis, it also forms a polarized single layer of cuboidal cells, but in contrast to

ectodermal epithelia, whose apical membranes face the outside world or a lumen, its apical membrane forms cell-cell contacts with the germline cells.

4. The neural stem cell (neuroblast, NB, see 2.3).

5. The neuron with an axon and dendrites.

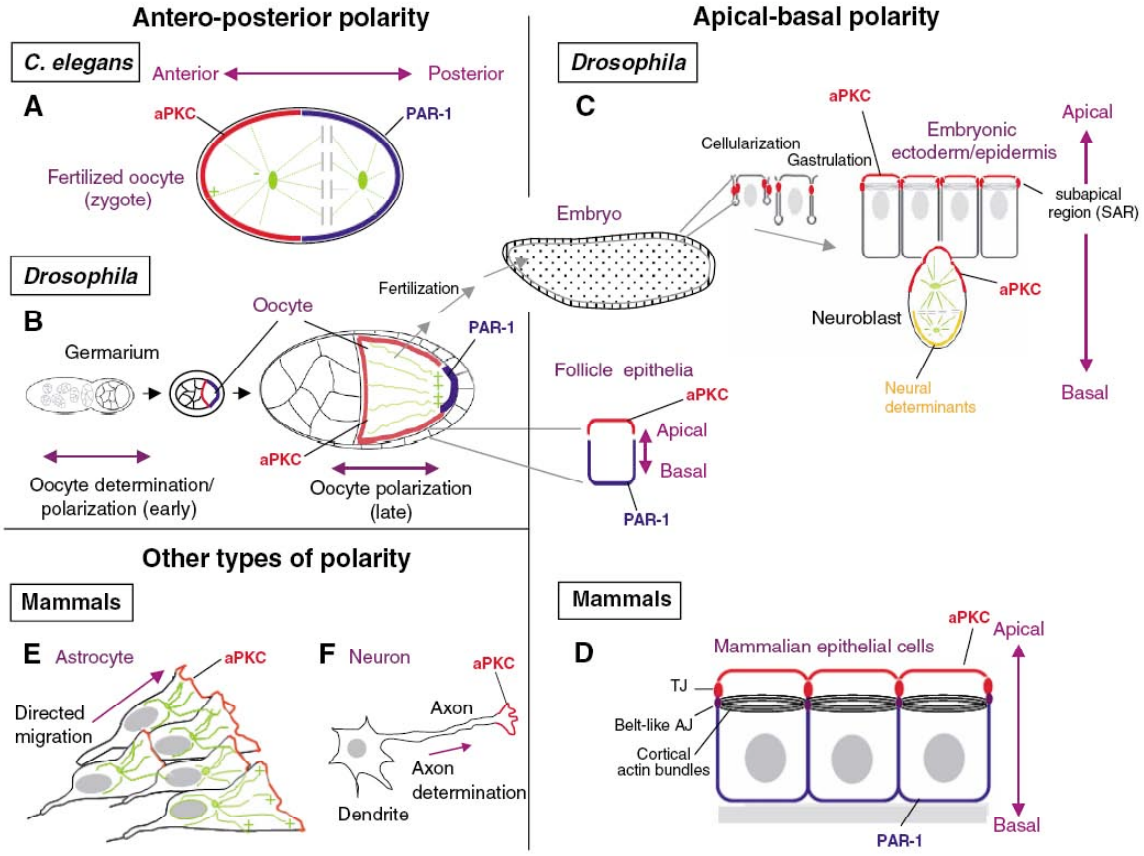


**Figure 1.** Various types of polarized cells (from Suzuki and Ohno 2006)

## 3.2. The *Drosophila* embryonic epidermis as a model for epithelial polarity

The ectodermal epidermis of the *Drosophila* embryo is a good model to study fundamental mechanism of cell polarity. The polarity is first established during blastoderm stage (ca. 2:10h after egg deposition), concomitantly with the invagination of the plasma membrane separating the syncytium (Lecuit, 2004). Compared with the mammalian cell culture system, it has been shown that many of the basic mechanisms and genes regulating epithelial polarity are highly conserved throughout evolution (Knust and Bossinger, 2002).

Polarity in epithelial cells is based on the segregation of proteins and lipids between an apical membrane domain, a lateral cell-cell contact zone and a basal cortex, which is in close contact to the underlaying tissue. The last two domains are often subsumed as the basolateral domain. One key step in the establishment and restriction of the membrane domains is the formation of specialized cell-cell contact zones.

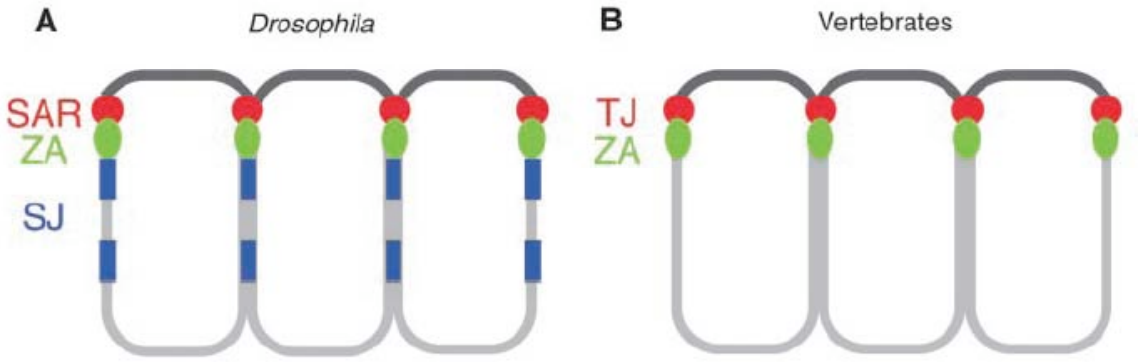


**Figure 2.** Junctional complexes of epithelial cells in vertebrates and *Drosophila* (from Knust and Bossinger 2002)

In vertebrates, adherens junctions between neighbouring cells are formed in the zonula adherens (ZA), a process which mainly involves the cadherin-catenin complex, Therefore, the transmembrane protein E-cadherin (or other members of the cadherin family) forms first cis-cellular and later trans-cellular dimers in a calcium dependent fashion (Nelson, 2008). By their intracellular domain, cadherins recruit β-catenin, which in turns bind to α-catenin which finally links the cadherin-catenin complex directly or viaa vinculin and α-actinin to the actin cytoskeleton (Nelson, 2008; Perez-Moreno et al., 2003). The correct formation of the ZA is a crucial prerequisite for the establishment of the tight junctions (TJ), which are located apical of the ZA and

composed of different protein complexes which finally act together to seal the intercellular space (Matter, 2000; Tsukita et al., 2001). Beside members of the transmembrane-protein families JAM (junctional adhesion molecule), claudin and occludin, there are also some cytoplasmic proteins localized to the TJ, namely the zonula occludens proteins (ZO-1-3), MAGI-proteins and the PAR/aPKC complex proteins (cp 2.4) (Tsukita et al., 2001). One more TJ complex, which is also conserved throughout evolution is the Crumbs (Crb) / PALS1 (protein associated with Lin7) / PATJ (PALS1-associated TJ protein) complex. As an antagonist to the apical junctional regulators functions the Discs Large (Dlg) / Scribble / Lethal (2) giant larvae (Lgl) complex at the basolateral domain.

The components of the AJ, its assembly and regulation is mostly conserved in from fly to man but in contrast to mammalian cells there is no real TJ in the *Drosophila* epithelium but a so called sub-apical region (SAR), which is located apical of the AJ. This junctional belt is predominately established by the transmembrane protein Crb and its intracellular binding partner Stardust, although components of the PAR/aPKC complex are also partly localized to the SAR and regulate SAR and AJ assembly (Bilder et al., 2003; Harris and Peifer, 2005; Knust and Bossinger, 2002). Analogue to mammalian epithelial cells, the Dlg complex is located at the basolateral membrane.

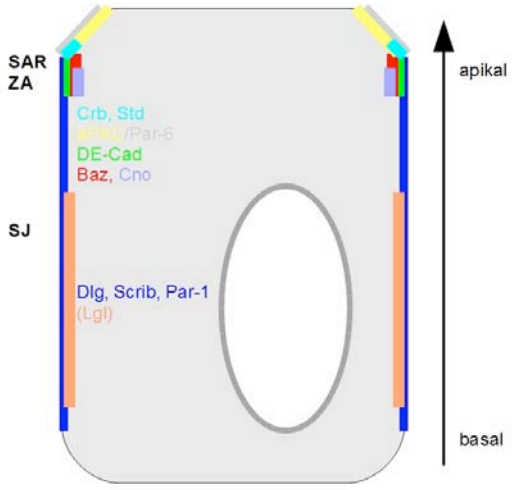


**Figure 3.** Localization of protein complexes in the *Drosophila* epithelium (Beati, personal communication)

## 3.3. The early development of the *Drosophila* nervous system

The development of the nervous system of *Drosophila* starts with the delamination of the NBs during stage 9 of embryogenesis (approximately four hours after egg deposition) from the overlying ectodermal epithelium in the so-called "neurogenic region". Prior to the first mitosis, apical-basal polarity is established, partly inherited from the epithelium (Wodarz, 2005; Wodarz and Huttner, 2003). In metaphase, members of the PAR/aPKC-complex (see below) are positioned at the apical membrane domain, together with the Insc/Pins/Gαi complex. In contrast, certain cell fate determinants like the transcription factor Prospero, the proteins Brain Tumor (Brat) and Numb and their adaptor proteins Miranda and Partner of Numb are localized to the basal cortex. Additionally, the spindle, which is first in parallel to the overlaying epithelium, rotates by 90° and upon unequal cytokinesis the NB divides asymmetrically into a bigger, apically localized daughter cell and a smaller, basally localized daughter cell. Proteins localized apically during metaphase are inherited by the bigger daughter cell, which retains stem cell abilities and undergoes more cycles of asymmetric cell division. In contrast, proteins targeted to the basal cortex in the dividing NB segregate exclusively into the smaller daughter cell, the so-called "ganglion mother cell" (GMC), which divides only once more, giving rise to two neurons or glial cells. The apical-basal polarity of the NB, which is coordinated with spindle orientation in metaphase, is crucial for asymmetric cell division and thereby also for the development of the nervous system: Loss of polarity often results in a symmetric division, generating two daughter cells with stem cell abilities that both continue to divide, eventually leading to tumor formation (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006; Wodarz and Näthke, 2007).

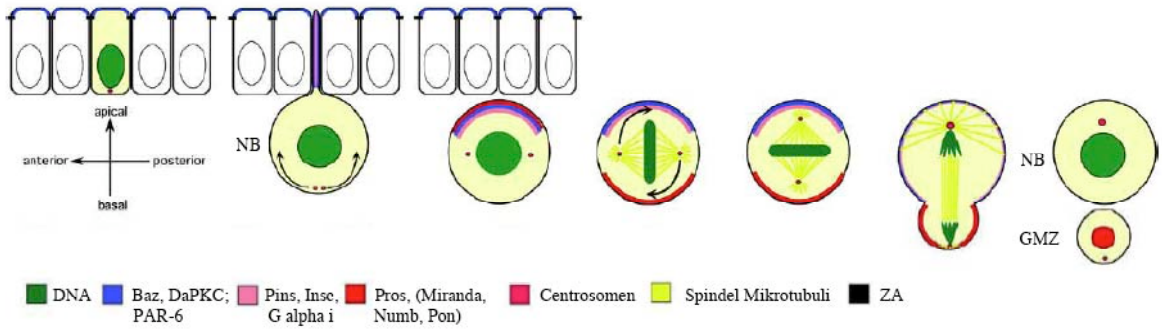


**Figure 4**. Delamination and asymmetric cell division in *Drosophila* NBs (Wodarz 2003).

## 3.4. The PAR-complex

One of the most important regulators of cell polarity is the PAR-aPKC- (partitioning-defective – atypical protein kinase C) complex. It is highly conserved throughout evolution from worm to man (Suzuki and Ohno, 2006) and consists of the scaffolding proteins PAR-3 (Bazooka, Baz in *Drosophila*) and PAR-6 and the serine-threonine kinase aPKC. This complex localizes to the apical cortex in epithelial cells and NBs and to the anterior cortex in the *C.elegans* zygote and the oocyte of *Drosophila* (Figure 1). It is antagonized by other PAR proteins, namely PAR-1, a serine-threonine kinase that localizes basolaterally in epithelia and posterior in the oocyte, and the adaptor protein PAR-5 (14-3-3ε and leonardo in *Drosophila*).

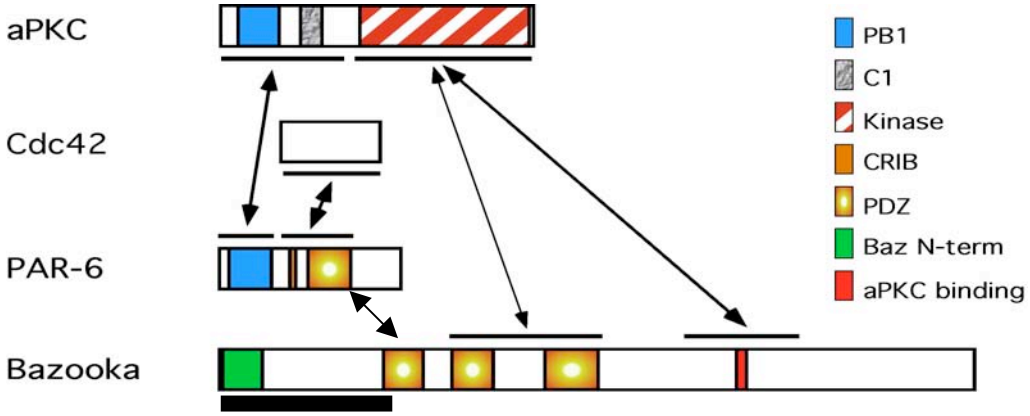


**Figure 5.** Interacting domains in the PAR-complex (adapted from Johnson and Wodarz 2003). Baz serves as a scaffold to recruit PAR-6 and aPKC to the cortex: The first PDZ domain of Baz interacts with the PDZ domain of PAR-6 and the aPKC binding domain with the kinase domain of aPKC. Additionally, aPKC can directly interact with PAR-6 via their PB1 (phagocyte oxidase/Bem1) domains.

## 3.5. Bazooka

The *bazooka* (*baz*) gene was first identified in a screen for embryonic patterning defects and obtained its name due to the big holes in the cuticle of *baz* mutant embryos (Wieschaus et al., 1984). *baz* encodes a large protein of 1464 amino acids that possesses three highly conserved PDZ-(**P**sd95, **D**isc large, **Z**O-1) domains and a conserved N-terminal oligomerization domain (CR1) (Kuchinke et al., 1998; Wieschaus et al., 1984) (Benton & St Johnston, 2003). Furthermore, for the mammalian and worm homologue of Baz, PAR-3, a conserved region of twenty amino acid residues has been described to interact with the kinase domain of aPKC (Izumi et al., 1998; Tabuse et al., 1998). In contrast, for Baz, the interaction with aPKC was mapped to the second and third PDZ domain (Wodarz et al., 2000).

PAR-6 can bind to the first PDZ domain of PAR-3 and additionally directly to aPKC (Joberty et al., 2000; Lin et al., 2000). In addition to these three "core" components of the PAR/aPKC complex, the small GTPase Cdc42 is often recruited into this complex. In fact, it can bind directly to PAR-6, regulating the binding affinity of the PAR-6-aPKC interaction and thereby aPKC kinase activity in various cell types of different species (Garrard et al., 2003; Joberty et al., 2000; Lin et al., 2000; Peterson et al., 2004). The specific contribution of Cdc42 to the function of the PAR-complex in the regulation of cell polarity still remains unclear, because Cdc42 is involved in several additional pathways connected with polarity.

Various studies have shown that the PAR complex and particularly Baz/PAR-3 acts at the top of a genetic hierarchy in the regulation of cell polarity (Johnson and Wodarz, 2003). Loss of Baz leads to a complete loss of cell polarity in most polarized cell types investigated so far. In fact, in *Drosophila*, Baz is one of the first apical cues in the ectodermal epithelium and it is essential for the establishment of the first adherens junctions during cellularization (Harris and Peifer, 2004). It is necessary for the correct targeting of Crumbs (Crb), a conserved transmembrane protein and key regulator of epithelial cell polarity, to the apical membrane (Harris and Peifer, 2004). In contrast, mutation of *crb* does not alter the apical localization of Baz in early embryogenesis (Bilder et al., 2003; Johnson and Wodarz, 2003). Moreover, Baz mediates assembly of the junctional protein complex of DE-cadherin (Drosophila E-cadherin) and Armadillo (the Drosophila homologue of β-catenin) (Harris and Peifer,

2004; Harris and Peifer, 2005). Consequently, loss of Baz results in an impaired assembly of the AJ.

In addition to epithelial polarity, the asymmetric cell division in embryonic and larval NBs is controlled by Baz (Knoblich, 2008; Wodarz, 2005). Here, Baz recruits Inscuteable (Insc) and Partner of Inscuteable (Pins) to the apical cortex, which in turn stabilizes the Baz protein (Schober et al., 1999; Wodarz et al., 1999). Like in epithelial cells, Baz also targets PAR-6 and aPKC to the apical cortex in dividing NBs (Petronczki and Knoblich, 2001; Wodarz et al., 2000). The apical accumulation of Baz is not affected upon loss of PAR-6 or aPKC, in contrast to the asymmetric localization of cell fate determinants, which ensure that only one daughter cell retains stem cell abilities (Petronczki and Knoblich, 2001; Rolls et al., 2003). This supports the hypothesis that Baz serves as a scaffold to ensure the correct localization and regulation of aPKC kinase activity (Wirtz-Peitz et al., 2008).

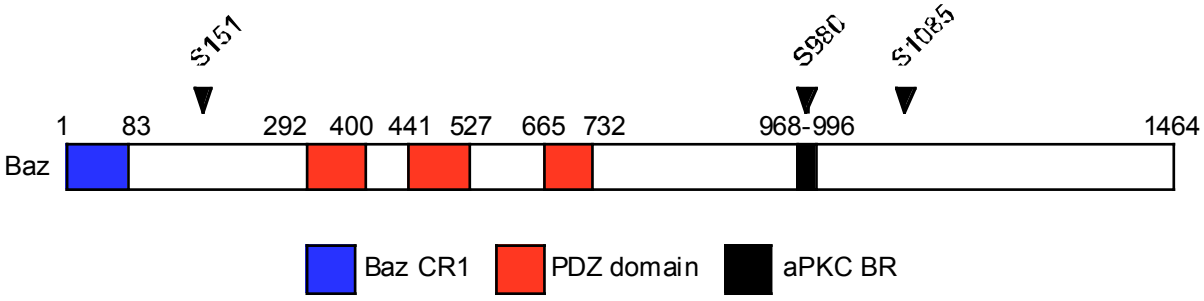


**Figure 6.** Structure of the Baz protein

Up to now, three conserved serine residues of Baz have been reported to be phosphorylated: serine 980 as mentioned above is phosphorylated by aPKC (Kim et al. submitted, (Nagai-Tamai et al., 2002). In a mammalian cell culture system, this phosphorylation has been shown to be crucial for the establishment but not for the maintenance of cell polarity (c.p. 3.3)(Nagai-Tamai et al., 2002).

Serine 151 and serine 1085 are phosphorylated by PAR-1, thus creating a binding site for 14-3-3 proteins (Benton and St Johnston, 2003). Furthermore, it has been demonstrated that the phosphorylation of Baz at these two sites cooperates in the exclusion of Baz from the lateral and basal membrane domain in the follicle epithelium and from the posterior cortex in the oocyte. Recently, a first genetic

interaction study suggested a role for PP2A as a counterpart of PAR-1 kinase activity in the development of the polarized photoreceptor cells (Nam et al., 2007).

## 3.6. Research objectives

Although various aspects of the function of Baz/PAR-3 in the control of cell polarity have been elucidated during the last decade, there are still many unanswered questions. One of the most intriguing problems is how exactly Baz is recruited to the membrane and how it is targeted to the apical membrane domain.

Therefore, the first aim of this study was to characterize the Baz protein functionally by a structural analysis using deletion constructs in transgenic flies and cell culture. From the subcellular localization of the mutated proteins conclusions can be drawn regarding the function of the different domains. This analysis was performed in four different polarized cell types, namely the ectodermal epidermis, the mesodermal follicle epithelium, the adult female germ line and the embryonic NBs.

Secondly, I analyzed the interaction between Baz and protein phosphatase 2A (PP2A), a potential interaction partner of Baz found in a yeast-two-hybrid screen. The focus of this project was to determine whether the potential dephosphorylation of three conserved serine residues in Baz by PP2A is required for the establishment and maintenance of cell polarity in NBs.

Finally, the phosphorylation of Baz by aPKC at the conserved serine 980, which has already been described to play an essential role in the establishment of cell polarity in mammalian epithelial cells (Nagai-Tamai et al., 2002), was elucidated by generation of mutations in this site and expression of the mutant constructs in flies and cell culture. The consequences of such mutations on cell polarity in different cell types and on the interaction between Baz and aPKC were characterized in detail.

# 4. Results

Every chapter within the results starts with a one-page description of:

• the main aim of the particular manuscript in the context of the complete thesis

• the authors and their contributions to the work, and

• the status of the manuscript.

## 4.1. Membrane targeting of Bazooka/PAR-3 is mediated by a novel phosphoinositide-binding domain

Within that project, various deletion constructs of Baz were expressed in different polarized tissues in the *Drosophila* embryo and adult female germ line using the UAS-GAL4 system. By indirect immunofluorescence and confocal laser microscopy, the subcellular localization of the mutated transgenes was investigated and its functionality was tested by a rescue experiment with two Baz NULL-alleles.

The potential lipid-binding capability of the PDZ domains and the C-terminus of Baz were tested using membrane lipids-strips.

**Michael P. Krahn**, Nannette Fischer and Andreas Wodarz

Author contributions to the work:

| | |
|---|---|
| **Michael P. Krahn**: | All experiments, besides*<br>writing of the manuscript |
| Nannette Fischer: | *Sequencing of the Baz alleles |
| Andreas Wodarz: | Editing of the manuscript |

**STATUS: SUBMITTED** to Current Biology

# Membrane targeting of Bazooka/PAR-3 is mediated by a novel phosphoinositide-binding domain

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**Summary**

Background

Cell polarity in higher animals is controlled by evolutionarily conserved protein complexes, which localize to the cytocortex in a polarized manner. The PAR-3/PAR-6/aPKC complex is the first to become asymmetrically localized and it controls the localization of additional complexes functioning further downstream in the regulation of cell polarity, including the Crumbs/Stardust/PATJ complex in epithelia and the Partner of Inscuteable/Gαi complex in neural precursor cells. The first component of the PAR-3/PAR-6/aPKC complex that is localized to the cortex is Bazooka/PAR-3 (Baz), a large scaffolding protein. How Baz is recruited to the membrane is unknown so far.

Results

Here we present a structure-function analysis of Baz focussing on its subcellular localization and function in four different polarized cell types of *Drosophila*: the ectodermal embryonic epidermis, the mesodermal follicle epithelium, embryonic neuoblasts and the oocyte. We show that the PDZ domains of Baz are dispensable for its correct localization, whereas a conserved region in the C-terminal part of Baz to which no function had been assigned so far is required and sufficient for membrane localization. This domain binds strongly to phosphoinositide membrane lipids and thus mediates cortical localization of Baz by direct interaction with the plasma membrane.

Conclusions

We have identified a novel phosphoinositide-binding domain that is necessary and sufficient for recruitment of Baz to the plasma membrane. Our findings reveal a mechanism for the coupling of plasma membrane polarity and cortical polarity.

**Introduction**

Baz/PAR-3 is a core component of the PAR-3/PAR-6/aPKC complex, which is conserved throughout evolution from worm to man [1, 2]. In a broad range of polarized cell types, the PAR-3/PAR-6/aPKC complex is required to define the axis of polarity: apical versus basal or anterior versus posterior [2]. During the past decade it became clear that Baz acts at the top of a hierarchy of molecules which are responsible for this polarization [3-5]. For example, in *C.elegans*, PAR-3 can localize to the anterior cortex in a PAR-6 and aPKC- independent fashion [6, 7]. In *Drosophila* neural precursor cells (neuroblasts, NBs), Baz does not only recruit aPKC and PAR-6 to the apical cortex, but also the Inscuteable/Pins/Gαi complex [8-13]. Similarly, in the *Drosophila* ectodermal epithelium, Baz serves as the first apical cue required for localization of Crumbs (Crb) to the apical membrane domain [14]. In all these cell types, loss of Baz/PAR-3 function leads to loss of cell polarity [10, 13, 15, 16].

These findings raise the question of how Baz itself is recruited to the membrane and how it obtains its polarized subcellular localization. This could be achieved in several ways, for instance by binding to an integral transmembrane protein, by binding to a membrane associated protein, by lipid modification or by direct binding to membrane lipids. Mammalian PAR-3 is recruited to tight junctions in epithelial cells by binding to the transmembrane protein Junctional Adhesion Molecule (JAM) via its first PDZ domain [17, 18]. However, there are no annotated homologs of JAM in *Drosophila*, ruling out this mechanism for membrane recruitment of Baz.

It was recently shown that the highly conserved second PDZ domain of rat PAR-3 binds to phosphoinositide lipids and is crucial for membrane association of PAR-3 in mammalian epithelial cells [19]. Binding to phosphoinositides is not a unique feature

of the second PDZ domain of PAR-3 but was also demonstrated for a variety of other PDZ domains [19-22]. Given the high conservation of Baz/PAR-3 throughout evolution, it is tempting to speculate that the second PDZ domain of Baz may be responsible for its membrane localization, but this has not been tested yet.

Additional evidence for the potential involvement of phosphoinositides in the localization of the PAR-3/PAR-6/aPKC complex has come from studies in cultured hippocampal neurons, where the phosphatidyl-inositol-3-kinase (PI3-kinase) pathway is required for the polarized localization of the complex to the tip of the axon [23]. Intriguingly, different phosphoinositides are restricted to different domains of the plasma membrane. Phosphatidylinositol (4, 5) bisphosphate ($PIP_2$) is restricted to the apical plasma membrane domain and Phosphatidylinositol (3, 4, 5) trisphosphate ($PIP_3$) to the basolateral domain in mammalian polarized epithelia [24, 25]. In *Drosophila* photoreceptor cells and ectodermal embryonic epithelia the distribution of $PIP_2$ and $PIP_3$ is reversed, with $PIP_3$ accumulating in the apical and $PIP_2$ in the basolateral membrane domain [26, 27]. The balance between $PIP_2$ and $PIP_3$ in the membrane is regulated by PI3-kinase and its antagonist, the lipid phosphatase PTEN [28]. PTEN directly binds to the third PDZ domain of Baz, revealing another important link between the PAR-3/PAR-6/aPKC complex and phosphoinositide signaling [19, 26, 27].

To address the mechanism of how Baz gets recruited to the plasma membrane and which domains of Baz are required for its function, we performed a structure-function analysis using a series of GFP-Baz fusion proteins lacking different regions of the protein. These GFP fusion proteins were expressed from UAS-driven transgenes in the embryonic epidermis, in embryonic NBs, in the follicle epithelium and in oocytes and their subcellular localization in these cell types was analyzed by confocal

microscopy. Furthermore, the mutant proteins were tested for their ability to rescue the lethality of strong loss-of-function mutations of *baz*.

Contrary to our expectations based on the study by Wu et al. [19], we found that deletion of the second or even of all three PDZ domains did not result in mislocalization of Baz, whereas a domain in the C-terminal region of Baz was necessary and sufficient for membrane targeting. We show that this domain binds strongly to phosphoinositides, in contrast to a protein fragment comprised of the three PDZ domains of Baz. Thus, our data reveal that Baz is recruited to the membrane by direct binding to phosphoinositides via a novel phosphoinositide-binding domain.

## Results

### Molecular analysis of loss-of-function alleles of *baz*

Baz is a large scaffolding protein of 1464 aa, but with the exception of the N-terminal conserved region 1 (CR1; Fig. 1), which is required for homo-dimerization [29], and two conserved phosphorylation sites for the kinase PAR-1 (S151 and S1085), which serve as binding sites for 14-3-3 proteins [30, 31], little is known about the importance of individual domains for the function and proper subcellular localization of Baz. To identify protein domains and individual amino acid residues that are crucial for the function of Baz, we sequenced four mutant *baz* alleles that were either induced by treatment with ethyl-methanesulfonate (EMS) ($baz^{Xi106}$; [32], $baz^{EH747}$; [33]) or by exposure to X-rays ($baz^{815\text{-}8}$; [34], $baz^{XR11}$; [35], R. Stanewsky, unpublished). Three alleles contained nonsense mutations giving rise to Baz proteins truncated in the N-terminal quarter of the protein that most likely are completely nonfunctional (Fig. 1A). In $baz^{XR11}$ we could not detect any mutation that would lead

to changes in the Baz protein sequence, pointing to a mutation in a regulatory element outside of the coding region in this allele.

Structure-function analysis of Baz using GFP-Baz fusion proteins

Because the molecular analysis of the four mutant alleles of *baz* did not allow us to draw any conclusions on the functional requirement for individual domains of Baz, we generated a series of constructs encoding full-length and deletion mutants of Baz tagged at the N-terminus with green fluorescent protein (GFP) (Fig. 1B). These constructs were expressed with the UAS-GAL4 system [36] in four different polarized cell types: In the embryonic epidermis using the ubiquitous driver daughterless::GAL4 (da::G4), in the adult follicle epithelium, which is derived from mesodermal stem cells by Cu::GAL4 (Cu::G4), in embryonic NBs using worniu::GAL4 (wor::G4) and in the adult female germ line with nanos::GAL4 (nos::G4), in order to test the requirement of the different domains of the Baz protein for proper subcellular localization in these cell types.

Full length GFP-Baz is fully functional as it rescued the embryonic lethality of two strong *baz* alleles ($baz^{815-8}$ and $baz^{XR11}$; Table 1), like untagged full length Baz [35]. Furthermore, the GFP-Baz fusion protein showed exactly the same subcellular localization as the endogenous Baz protein in all cell types that we analyzed (Fig. 2). In embryonic epidermal cells and in the follicle epithelium, full-length GFP-Baz localized to the ZA and colocalized there with DE-Cadherin (DE-Cad) but not with Discs large (Dlg), a marker for the basolateral membrane (Fig. 2A, B). In embryonic NBs, GFP-Baz formed an apical crescent at pro- and metaphase and colocalized with aPKC, whereas Miranda (Mira) formed a crescent at the basal cortex (Fig. 2C). In

stage 10 oocytes GFP-Baz formed a gradient at the membrane with high concentrations anterior and low concentrations at the posterior pole (Fig. 2D).

The N-terminal region of Baz is required for polarized localization in NBs and oocytes

The N-terminal region of Baz (CR1; Fig. 1) is highly conserved and is predicted to adopt a three-dimensional structure similar to that of the *E. coli* protein DinI and a homologous protein in coliphage 186. Baz CR1 is required for homophilic di- or oligomerization of the Baz protein [29]. Furthermore, Baz CR1 was found to be necessary but not sufficient for apical membrane localization of a Baz-GFP fusion protein in the follicle epithelium [29]. We investigated the subcellular localization of a mutant GFP-Baz fusion protein lacking the first 317 amino acids including CR1 (GFP-BazΔ1-317, Fig. 1B). In the embryonic epidermis GFP-BazΔ1-317 colocalized with endogenous Baz (data not shown) and with DE-cad in the ZA (Fig. 3A). In the follicle epithelium, the subcellular localization of GFP-BazΔ1-317 was dependent on the level of overexpression. At low levels of overexpression, GFP-BazΔ1-317 was targeted correctly to the ZA and colocalized with DE-Cad (Fig. 3B). In cells containing higher levels of the protein, GFP-BazΔ1-317 was partly diffusely distributed in the cytosol (Fig. 3B, arrows) but still a considerable amount of protein accumulated in the ZA.

In embryonic NBs, GFP-BazΔ1-317 was associated with the membrane but localized to the entire cortex instead of forming an apical crescent (Fig. 3C). GFP-BazΔ1-317 also localized homogeneously to the entire cortex of the oocyte (Fig. 3D), demonstrating that the N-terminal region of Baz is required for its polarized localization in these two non-epithelial cell types.

The PDZ domains of Baz are not required for membrane targeting

For rat PAR-3 (also called atypical protein kinase C specific interacting protein, ASIP) [37], the second PDZ domain was shown to interact with phosphoinositide membrane lipids [19]. This interaction depends on a PIP head group polar binding pocket and a positively charged cluster of amino acids in the second PDZ domain of PAR-3. Deletion of the second PDZ domain or mutation of single conserved amino acid residues involved in lipid binding was reported to result in a total loss of membrane association of PAR-3 in MDCK cells [19]. To test whether the second PDZ domain has a similar function in *Drosophila* Baz, we expressed deletion mutants of Baz lacking individual PDZ domains (GFP-BazΔPDZ1/2/3), or all three PDZ domains together (GFP-BazΔPDZ1-3) (Fig. 1B) in *Drosophila* embryonic and adult tissues. Neither deletion of any single PDZ domain (data not shown), nor deletion of all three PDZ domains resulted in a disturbed localization of the respective mutant Baz protein compared to wild type Baz (Suppl. Fig. 1).

One explanation could be that correctly localized endogenous Baz protein localizes the mutant protein via its N-terminal oligomerization domain [29]. However, in hemizygous $baz^{815-8}$ mutant embryos the GFP-BazΔPDZ1-3 protein still localized normally at late embryonic stages when the maternally contributed endogenous wild type Baz protein had already disappeared (data not shown). Additionally, in S2R+ cells, which express only low levels of endogenous Baz, GFP-BazΔPDZ1-3 was correctly targeted to the membrane indistinguishable from its wild type counterpart (Suppl. Fig. 2B).

These results show that the correct subcellular localization of Baz in the four cell types investigated here is independent of its PDZ domains. Consistent with this

conclusion, fragments of Baz containing all three PDZ-domains but lacking portions of the C-terminal region did neither show any significant membrane localization in the embryonic epidermis or in follicle cells nor in S2R+ cells (see below). This further suggests that the membrane binding ability of the PDZ domains of Baz is not sufficient to link Baz to the membrane in vivo. Nonetheless, the PDZ domains 1 and 2 are essential for the function of Baz, because mutant forms of Baz lacking these domains fail to rescue the lethality of strong *baz* loss-of-function alleles (Table 1).

Truncation of the C-terminal region of Baz abolishes membrane association

In contrast to Baz CR1, the three PDZ domains and the phosphorylation sites for aPKC and PAR-1 (S980 and S151/S1085 respectively), the C-terminal region of Baz is quite divergent from vertebrate and *C. elegans* PAR-3. To assess the function of this part of Baz, we generated a series of constructs encoding proteins with C-terminal truncations (Fig. 1B). Deletion of the non-conserved potential PDZ binding motif at the very C-terminus of Baz (SEVL; GFP-BazΔ1461-1464) did not affect the normal subcellular localization of Baz (data not shown). GFP-BazΔ1325-1464 (data not shown) and GFP-BazΔ1222-1464 were also localized correctly in all tissues analyzed (Suppl. Fig. 3). Deletion of 349 or 463 aa from the C-terminus (GFP-BazΔ1097-1464 and GFP-BazΔ1001-1464, respectively) led to increased accumulation of the truncated protein in the cytoplasm (Fig. 4A – C; Suppl. Fig. 4A). Only a small fraction of these mutant Baz proteins was still localized correctly to the ZA in epithelial cells (Fig. 4A) and to the apical cortex in NBs (Fig. 4C). Deletion of 496 aa from the C-terminus (GFP-BazΔ969-1464) completely abolished membrane localization of Baz in all tissues analyzed (data not shown).

In S2R+ cells, which only express low levels of endogenous Baz protein, GFP-BazΔ1097-1464 and GFP-BazΔ1001-1464 were homogeneously distributed in the cytosol without accumulation at the cell cortex (Suppl. Fig. 2E, F). In contrast, GFP-Baz proteins with smaller truncations were correctly targeted to the membrane (Suppl. Fig. 2C, D).

We assessed the functionality of the different variants of Baz with C-terminal truncations by rescue experiments. Deletion of up to 367 aa from the C-terminus allowed rescue of the lethality of $baz^{815-8}$ and of $baz^{XR11}$ to the adult, whereas larger deletions eliminating the conserved regions surrounding the phosphorylation sites for PAR-1 and aPKC did not rescue (Table 1).

A domain close to the C-terminus of Baz is sufficient for localization to the plasma membrane

From our experiments we conclude that the C-terminal region is required for membrane localization of Baz in different polarized tissues of the fly. In order to test whether the isolated C-terminal region is sufficient for membrane binding of Baz, we generated transgenic flies expressing a GFP-tagged C-terminal fragment of Baz (aa 905-1464, GFP-BazΔ1-904). The truncated protein was targeted to the plasma membrane in the epidermis of stage 12 embryos and showed partial colocalization with endogenous Baz protein (data not shown) and DE-Cad (Fig. 4D; Suppl. Fig. 7A). Only a small fraction of GFP-BazΔ1-904 was mislocalized to the cytosol (Fig. 4D; Suppl. Fig. 7A). In S2R+ cells, GFP-BazΔ1-904 was localized to the cortex (Suppl. Fig. 2G), consistent with the hypothesis that the C-terminal region of Baz contains a membrane-targeting domain.

In the follicle epithelium, GFP-BazΔ1-904 localized to the entire apical and lateral membrane and to the cytosol, instead of being restricted to the ZA (Fig. 4E). In embryonic NBs, GFP-BazΔ1-904 was still localized at the membrane but did not form an apical crescent (Fig. 4F). This underlines our previous finding that the N-terminal region of Baz is required for correct apical targeting in embryonic NBs (see above). In the oocyte, GFP-BazΔ1-904 was cytosolic and did not show any membrane localization (Suppl. Fig. 4B).

Because a truncation of 243aa (GFP-BazΔ1222-1464) still allowed correct localization of Baz to the ZA in the embryonic epidermis (Suppl. Fig. 3), we tested whether an even smaller C-terminal region is able to associate with the membrane. Therefore we expressed aa 905-1221 of Baz as a GFP fusion protein (Fig. 1B; GFP-BazΔ1-904Δ1222-1464). GFP-BazΔ1-904Δ1222-1464 localized to the plasma membrane, similar to GFP-BazΔ1-904 and also localized to the cortex in S2R+ cells (data not shown; Suppl. Fig. 2H).

Within the region from aa 905-1221, which is sufficient for membrane association of Baz, three regions are highly conserved between Baz and vertebrate PAR-3 (Suppl. Fig. 5): 1) the aPKC-binding domain (aa 971-985) including the phosphorylation site for aPKC (S980) [7, 37, 38], 2) the binding site for 14-3-3/PAR5 proteins (aa 1073-1093), which contains a phosphorylation site for PAR-1 (S1085) [30, 39, 40] (Krahn et al. in revision) and 3) a 20 aa stretch (aa 1173-1193) with up to now unknown function.

Deletion of the aPKC-binding domain (GFP-BazΔ968-996) did not affect correct localization of Baz in the epidermis (Suppl. Fig. 6A) nor in embryonic NBs (Suppl. Fig. 6D). In the follicle epithelium at stage 6, the mutated Baz was correctly targeted

to the ZA (Suppl. Fig. 6B), whereas at stage 10 it accumulated in dot-like structures in the cytosol (Suppl. Fig. 6C). In the oocyte, only a faint membrane staining was detectable, most of the protein accumulated in aggregates in the cytoplasm (Suppl. Fig. 6E). Variants of Baz deleted for the binding site for 14-3-3/PAR5 proteins (GFP-BazΔ1073-1093) or the conserved stretch of aa with unknown function (GFP-BazΔ1173-1193) localized normally in epithelia and NBs (data not shown).

To investigate the function of the three conserved sequence blocks within the region sufficient for membrane localization of Baz (aa 905-1464), we generated constructs comprising aa 905-1464 of Baz with the corresponding small internal deletions (Fig. 1B). GFP-BazΔ1-904Δ968-996 and GFP-BazΔ1-904Δ1073-1093 localized to the membrane similar to GFP-BazΔ1-904 (Suppl. Fig. 7A - C). In contrast, GFP-BazΔ1-904Δ1173-1193 did not show any membrane localization and was completely cytosolic (Suppl. Fig. 7D), demonstrating that in the absence of the N-terminal 904 aa, the conserved sequence block from aa 1173-1193 is essential for membrane localization.

The C-terminal region of Baz binds to phosphoinositides

Attachment of a cytoplasmic protein to the plasma membrane can be achieved either by binding to a transmembrane or membrane-associated protein or by direct anchorage to the lipid bilayer of the membrane. The latter can be mediated by posttranslational protein modification, e.g. prenylation and palmitoylation [41, 42] or by lipid binding domains, such as PH, FYVE and PX domains [43]. Sequence analyses using the BLAST and SMART algorithms did not reveal the existence of any known lipid-binding domain in the C-terminal region of Baz.

To elucidate whether the C-terminal region of Baz, which is necessary and sufficient for membrane association as shown here, binds to either a transmembrane or membrane associated protein, we performed a yeast-two-hybrid screen with aa 725-1464 of Baz as bait. After screening of 225 mio interactions, we did not find any interaction with a transmembrane or membrane associated protein that might serve as a linker to the membrane (data not shown). We then performed lipid-binding assays with two fragments (amino acids 905-1221 and 947-1464) of Baz fused to glutathione-S-transferase (GST) (GST-Baz905-1221 and GST-Baz947-1464). Both fusion proteins bound strongly to PI(4, 5)$P_2$ and PI(3, 4, 5)$P_3$, in contrast to a GST fusion protein containing all three PDZ domains of Baz (GST-BazPDZ1-3) and GST alone (Fig. 5).

These findings show that the C-terminal region from aa 947-1221 of Baz is sufficient for membrane association, raising the question of whether replacement of this region for an unrelated phosphoinositide binding domain can restore proper localization of Baz in different cell types. To answer that question, we generated transgenic flies expressing a chimeric protein (GFP-BazΔ1107-1464PHP; Fig. 1B) consisting of the first 1106 aa of Baz and the pleckstrin homology (PH) domain of human phospholipase Cδ, which binds specifically to PI(4, 5)$P_2$ [44]. GFP-BazΔ1107-1464PHP was localized correctly to the ZA in the embryonic epidermis (Fig. 4G). In the follicle epithelium, GFP-BazΔ1107-1464PHP was localized to the plasma membrane, but instead of being strongly enriched at the ZA, the protein was found at the free apical membrane and to a lesser extent along the lateral membrane (Fig. 4H). GFP-BazΔ1107-1464PHP was targeted correctly to the apical cortex in embryonic NBs (Fig. 4I) but was not excluded form the posterior oocyte cortex at stage 10 (Suppl. Fig. 4C). Essentially the same subcellular

localization was observed for a variant of Baz (GFP-BazΔ1107-1464PHS; Fig. 1B) in which aa 1108-1464 were replaced by the PH domain of the protein Stepke [45], which specifically binds to PI(3, 4, 5)$P_3$ (data not shown). These findings suggest that the binding to phosphoinositides as such is sufficient for localization of Baz to the membrane, and that the specific localization to the ZA and to the apical membrane domain is mediated by domains located in the N-terminal half of the protein.

**Discussion**

In all higher animals, cell polarity in a wide variety of cell types is controlled by the activity of the PAR-3/PAR-6/aPKC complex. PAR-3/Baz is the first component of this complex to become asymmetrically localized to the cortex underlying the plasma membrane, raising the question of how PAR-3/Baz is anchored at the membrane. So far, no transmembrane protein has been identified as a direct binding partner of Baz. Baz could be indirectly associated with the transmembrane protein Crb, since both Baz and Crb can bind to PAR-6 [12, 46-49]. However, indirect binding of Baz to Crb could only explain the membrane localization of Baz in epithelial cells and not in NBs or S2R cells, where Crb is not expressed. Furthermore, Baz is already localized to the membrane before Crb expression starts and Baz is positioned normally at the ZA in *crb* mutant embryonic epithelia, indicating that Crb cannot be responsible for membrane localization of Baz [5, 50]. Baz has also been reported to bind Armadillo (Arm), the *Drosophila* homolog of beta-catenin, which binds to the cytoplasmic tail of cadherins [51]. However, this interaction cannot be responsible for membrane recruitment of Baz, because Baz localization to the membrane is independent of the formation of E-cadherin-dependent cell-cell-contacts [50]. Moreover, deletion of the

first PDZ domain of Baz, which mediates binding to Arm, does not affect membrane localization of Baz.

The N-terminal conserved region 1 (CR1) is responsible for the homodimerization of Baz and PAR-3 [29, 52]. A mutant Baz-GFP fusion protein lacking CR1 localized to the cytoplasm in follicle cells, instead of being localized to the apical membrane and the ZA like wild type Baz [29]. In our hands, the localization of GFP-Baz lacking CR1 (GFP-BazΔ1-317) in follicle cells was dependent on the level of overexpression. At low levels of overexpression, most of the mutant protein was correctly localized to the ZA and to the apical membrane, and only upon stronger overexpression the mutant protein was partly mislocalized to the cytoplasm. In the embryonic epidermis, the localization of GFP-BazΔ1-317 was indistinguishable from wild type Baz, demonstrating that CR1 is dispensable for proper localization of Baz in this tissue. In contrast, GFP-BazΔ1-317 was localized uniformly around the cortex in neuroblasts and in the oocyte, revealing that aa 1-317 are required for the exclusion of Baz from the basal neuroblast cortex and the posterior oocyte cortex. At present we do not know whether these defects are due to compromised oligomerization or due to other, up to know unknown functions of the N-terminal region of Baz. In addition to CR1, the region deleted in GFP-BazΔ1-317 contains S151, a phosphorylation target for the kinase PAR-1, which localizes to the posterior oocyte cortex and the basal neuroblast cortex and destabilizes Baz at these sites [30, 31]. However, we do not think that deletion of S151 is responsible for the mislocalization of the GFP-BazΔ1-317 fusion protein, because a point mutation changing S151 to A does not significantly affect the localization of GFP-Baz [30] (MPK and AW, unpublished).

The PDZ domains of mammalian PAR-3 have been implicated in membrane targeting by two different mechanisms. The first PDZ domain of rat PAR-3 binds to the C-

terminus of junctional adhesion molecule 1 (JAM-1), a transmembrane protein localized at the tight junction [17]. The second PDZ domain of rat PAR-3 was shown to bind phosphoinositides and deletion of this domain led to cytoplasmic localization of the mutant PAR-3 in MDCK II epithelial cells [19]. However, deletion of all three PDZ domains of mouse PAR-3 did not affect its localization to the tight junction in MDCK cells [52], questioning the functional significance of phosphoinositide binding by the second PDZ domain of PAR-3. In *Drosophila*, deletion of individual PDZ domains or of all three PDZ domains together did neither affect the membrane localization of Baz per se, nor the asymmetric localization of Baz in any of the four cell types that we analyzed in this study. A GST fusion protein comprising all three PDZ domains of Baz showed weak if any binding to phosphoinositides in vitro, suggesting that this functional feature may not be shared between flies and mammals. Nonetheless, with the exception of PDZ domain 3, which appears to be dispensable for development of the fly, deletion mutants lacking the first or second PDZ domain of Baz were not capable of rescuing strong *baz* loss-of-function mutations, demonstrating an essential function for these two PDZ domains unrelated to membrane targeting, presumably by recruiting distinct interaction partners to the membrane.

Here we have shown that membrane localization of Baz depends on the region between aa 947-1221 containing the aPKC target site S980, the PAR-1 target site S1085 and a third conserved stretch of amino acids (aa 1173-1193) to which no function had been assigned so far. A fusion protein of this region with GST binds strongly to phosphoinositide membrane lipids in vitro. The same region fused to GFP is sufficient to target the fusion protein to the membrane in epithelia and neuroblasts of transgenic animals. However, in contrast to full-length Baz, this fusion protein does

not get asymmetrically localized in the four cell types we analyzed. A detailed deletion analysis of this lipid-binding region revealed that all three conserved sequence blocks are dispensable for proper localization of Baz when deleted individually in the context of the full-length protein, but that aa 1173-1193 are essential for membrane localization of the smaller C-terminal fragment of Baz. Our findings are consistent with a previous report showing that the region between aa 937-1024 of mouse PAR-3 (corresponding to aa 1124-1188 in Baz) is required for localization to the tight junction in MDCK cells [52].

**Conclusions**

We have shown that the proper asymmetric localization of Baz in four different cell types of *Drosophila* generally involves two separate mechanisms. A novel phosphoinositide-binding domain in the C-terminal region of Baz is responsible for the recruitment of Baz to the plasma membrane. In addition to this lipid-binding domain, the N-terminal 317 aa, which mediate homodimerization of Baz/PAR-3 [29, 52], are required for the asymmetric localization of Baz in NBs and the oocyte. This finding suggests that Baz may have to form higher order complexes in order to localize asymmetrically at the membrane.

**Experimental Procedures**

Fly stocks and genetics

The following alleles of *baz* were used in this study: $baz^{Xi106}$ [32], $baz^{815\text{-}8}$ [34], $baz^{EH747}$ [33] and $baz^{XR11}$ [35], (R. Stanewsky, unpublished). Transgenic flies carrying UAS::GFP-Baz constructs were generated using standard germ line transformation. The following GAL4 driver lines were used for expression of the transgenes in

different tissues: daughterless::GAL4 (da::G4) [53], Cu::GAL4, worniu::GAL4 (wor::G4), nanos::GAL4 (nos::G4). If not indicated otherwise, fly stocks were obtained from the Bloomington *Drosophila* stock center at the University of Indiana.

Immunohistochemistry

Embryos and ovaries were fixed in 4% formaldehyde, phosphate buffer pH 7.4. The primary antibodies used were rabbit anti Baz (1:1000) [10], rat anti Baz (1:500) [10], guinea-pig anti Mira (1:1000; Kim et al. submitted), rabbit anti PKCζ C20 (1:1000; Santa Cruz Biotechnology, Inc.), rat anti DE-Cadherin DCAD2 (1:50; Developmental Studies Hybridoma Bank, DSHB), mouse anti Dlg 4F3 (1:50; DSHB), rabbit anti Staufen (1:1000) [54], mouse anti Gurken 1D12 (1:10, DSHB), mouse anti GFP 3E6 (1:1000; Invitrogen). DNA was stained with DAPI (Invitrogen). Secondary antibodies conjugated to Cy2 and Cy3 were obtained from Jackson Laboratories. Secondary antibodies conjugated to Alexa 647 were obtained from Invitrogen. Images were taken on a Zeiss LSM 510 Meta confocal microscope and processed using Adobe Photoshop.

Lipid binding assays

Fusion proteins of different regions of Baz with GST were expressed in *E. coli* and affinity-purified according to the manufacturers instructions (Roche). Lipid strips containing spots of different membrane lipids (Echelon Inc) were then incubated with the purified GST-Baz fusion proteins according to the manufacturers instructions, washed and probed with antibodies against GST (SIGMA G7781) according to standard Western blot procedures.

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**Figure Legends**

Figure 1. Structure-function analysis of Baz. (A) Structure of the Baz protein. The positions of identified point mutations in three *baz* alleles are indicated by arrowheads. (B) Schematic representation of deletion mutants of Baz. All versions of Baz were N-terminally tagged with GFP and were expressed under control of the UAS-GAL4 system in transgenic flies and tissue culture cells. The amino acid residues still present in the deletion mutants are given in numbers at the borders of the deletions.

Figure 2. Subcellular localization of wild type GFP-Baz. (A) In the embryonic epidermis at stage 12, GFP-Baz (GFP) colocalizes with DE-Cadherin (DE-Cad) at the ZA, but does not overlap with basolateral Discs Large (Dlg). (B) In the follicle epithelium at stage 10 of oogenesis, GFP-Baz also colocalizes with DE-cadherin and is excluded from the basolateral membrane. (C) In embryonic metaphase neuroblasts (arrow), GFP-Baz colocalizes with aPKC in an apical cortical crescent opposite to the basal crescent of Miranda (Mira). (D) In stage 10 oocytes, GFP-Baz localizes to the cortex but is excluded from the posterior tip of the oocyte, marked by the presence of Staufen (Stau). The anterior-dorsal region of the oocyte is marked by the Gurken (Grk) protein. Genotypes are indicated in the respective panels. oc, oocyte, nc, nurse cell. DNA was stained with DAPI. Scale bars = 10 µm. In (A – C) apical is up. In (D) anterior is to the left.

Figure 3. Subcellular localization of GFP-BazΔ1-317. (A) In the embryonic epidermis at stage 12, the subcellular localization of GFP-BazΔ1-317 is indistinguishable from full-length wild type GFP-Baz (cf. Fig. 2A). (B) In the follicle epithelium at stage 10 of oogenesis, GFP-BazΔ1-317 colocalizes with DE-cadherin at the ZA and is

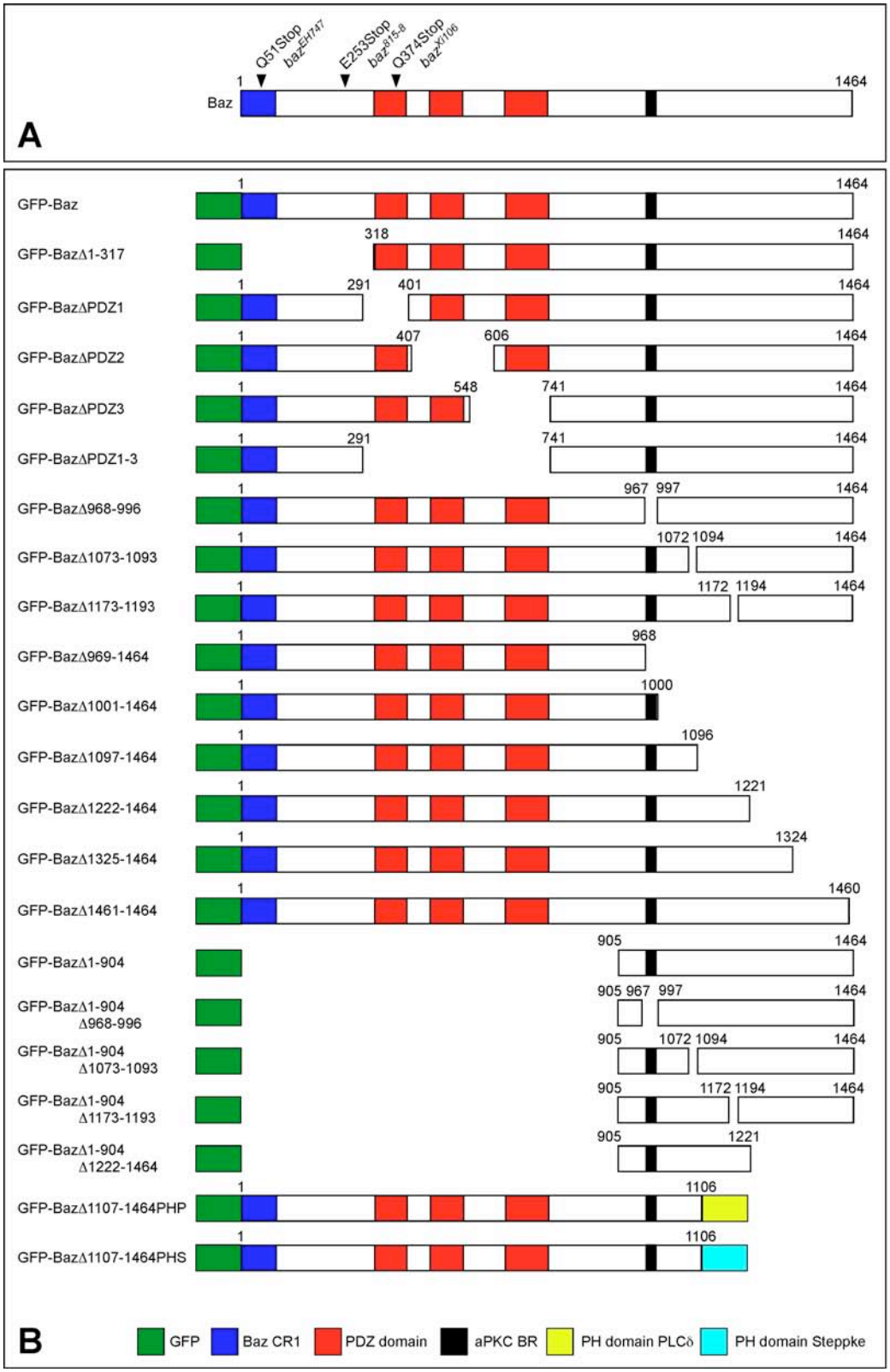
excluded from the basolateral membrane. Only few cells show increased staining in the cytoplasm (arrows). (C) In embryonic metaphase neuroblasts (arrow), GFP-BazΔ1-317 localizes all around the cortex. (D) In stage 10 oocytes, GFP-BazΔ1-317 localizes all around the cortex and is not excluded from the posterior tip of the oocyte, marked by the presence of Staufen. Genotypes are indicated in the respective panels. oc, oocyte, nc, nurse cell. DNA was stained with DAPI. Scale bars = 10 μm. In (A – C) apical is up. In (D) anterior is to the left.

Figure 4. The C-terminal region of Baz is necessary and sufficient for membrane localization. (A – C) GFP-BazΔ1097-1464 lacking 367 aa of the C-terminal region of Baz shows strongly reduced membrane localization and accumulates in the cytoplasm in the epidermis (A), in the follicle epithelium (B) and in neuroblasts (C, arrow). (D – F) GFP-BazΔ1-904 lacking CR1 and all PDZ domains localizes to the membrane but does not accumulate apically in the epidermis (D), in the follicle epithelium (E) and in mitotic neuroblasts (F, arrow). (G – I) Replacement of the C-terminal 357 aa of Baz by the pleckstrin homology (PH) domain of phospholipase C δ, which binds to PI(4, 5)$P_2$ leads to normal localization of the GFP-BazΔ1107-1464PH fusion protein in the epidermis (G), and in mitotic neuroblasts (I, arrow). In the follicle epithelium, the localization of GFP-BazΔ1107-1464PH is not restricted to the ZA but spreads along the apical and lateral membrane. Genotypes are indicated to the left and to the top of the respective image panels. Scale bars = 10 μm. Apical is up in all panels.

Figure 5. The region between aa 947-1221 of Baz binds to phosphoinositides. Lipid membrane strips were incubated with the GST fusion proteins indicated at the bottom and bound proteins were detected with anti GST antibody.

| **Construct** | **Rescue *baz*$^{815-8}$** | **Rescue *baz*$^{XR11}$** |
|---|---|---|
| GFP-Baz | + | + |
| GFP-BazΔ1-317 | - | - |
| GFP-BazΔPDZ1 | - | - |
| GFP-BazΔPDZ2 | - | - |
| GFP-BazΔPDZ3 | + | + |
| GFP-BazΔPDZ1-3 | - | - |
| GFP-BazΔ968-996 | - | - |
| GFP-BazΔ1073-1093 | | |
| GFP-BazΔ1173-1193 | + | + |
| GFP-BazΔ969-1464 | - | - |
| GFP-BazΔ1001-1464 | - | - |
| GFP-BazΔ1097-1464 | + | + |
| GFP-BazΔ1222-1464 | + | + |
| GFP-BazΔ1325-1464 | + | + |
| GFP-BazΔ1461-1464 | + | + |
| GFP-BazΔ1-904 | - | - |
| GFP-BazΔ1107-1464PHP | + | + |
| GFP-BazΔ1107-1464PHS | | |

Table 1. Rescue of the lethality of two strong *baz* alleles by GFP-Baz fusion proteins expressed with the UAS-GAL4 system under control of the da::GAL4 driver line. (+) indicates that rescued adult hemizygous *baz* mutant males were obtained that expressed the respective GFP-Baz transgene.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baz^815-8
Q374Stop
baz^X106
Baz
1
1464
B
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GFP-BazΔ1-317
GFP-BazΔPDZ1
GFP-BazΔPDZ2
GFP-BazΔPDZ3
GFP-BazΔPDZ1-3
GFP-BazΔ968-996
GFP-BazΔ1073-1093
GFP-BazΔ1173-1193
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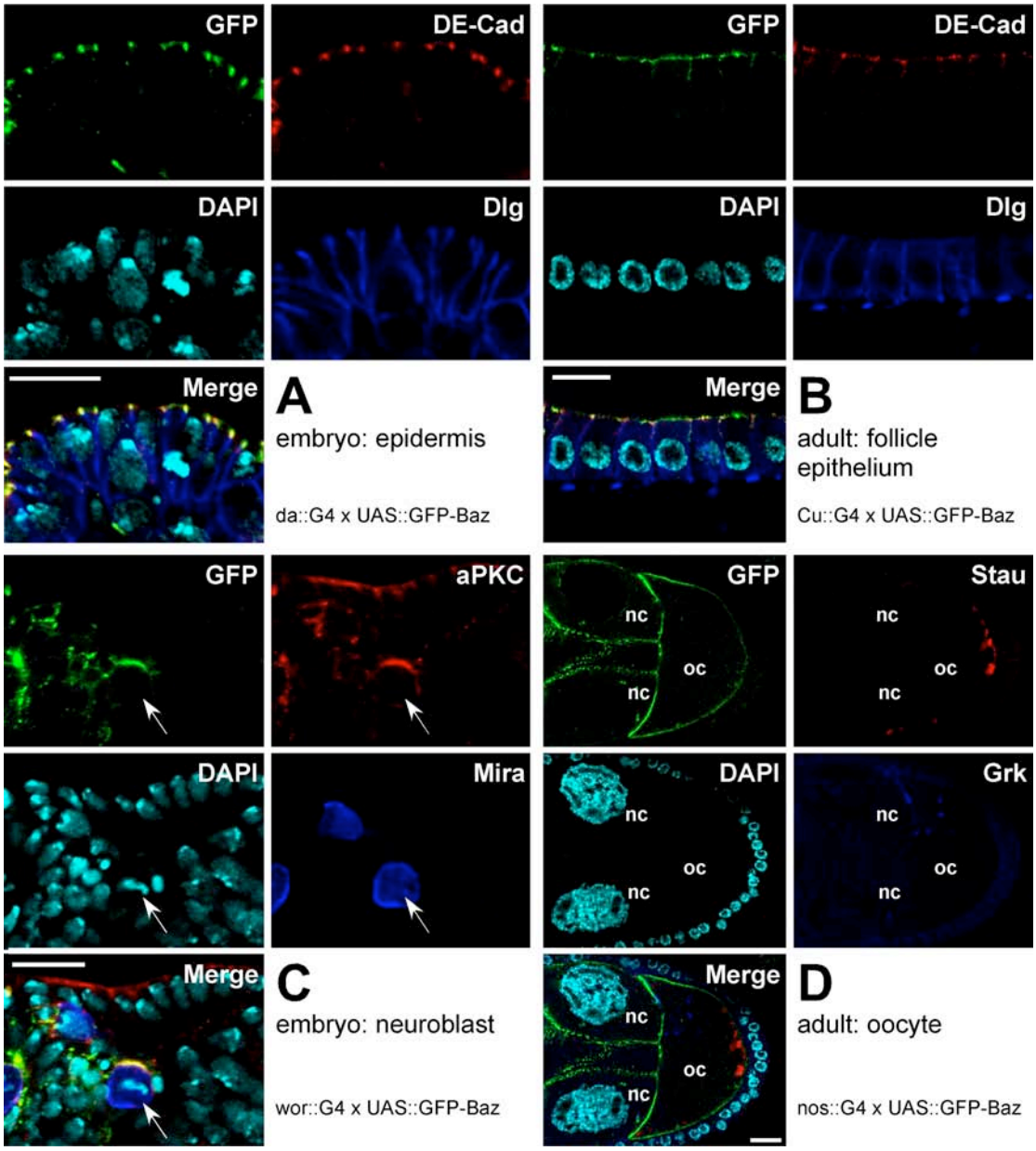
GFP-BazΔ1325-1464
GFP-BazΔ1461-1464
GFP-BazΔ1-904
GFP-BazΔ1-904 Δ968-996
GFP-BazΔ1-904 Δ1073-1093
GFP-BazΔ1-904 Δ1173-1193
GFP-BazΔ1-904 Δ1222-1464
GFP-BazΔ1107-1464PHP
GFP-BazΔ1107-1464PHS
GFP
Baz CR1
PDZ domain
aPKC BR
PH domain PLCδ
PH domain Steppke

Figure 1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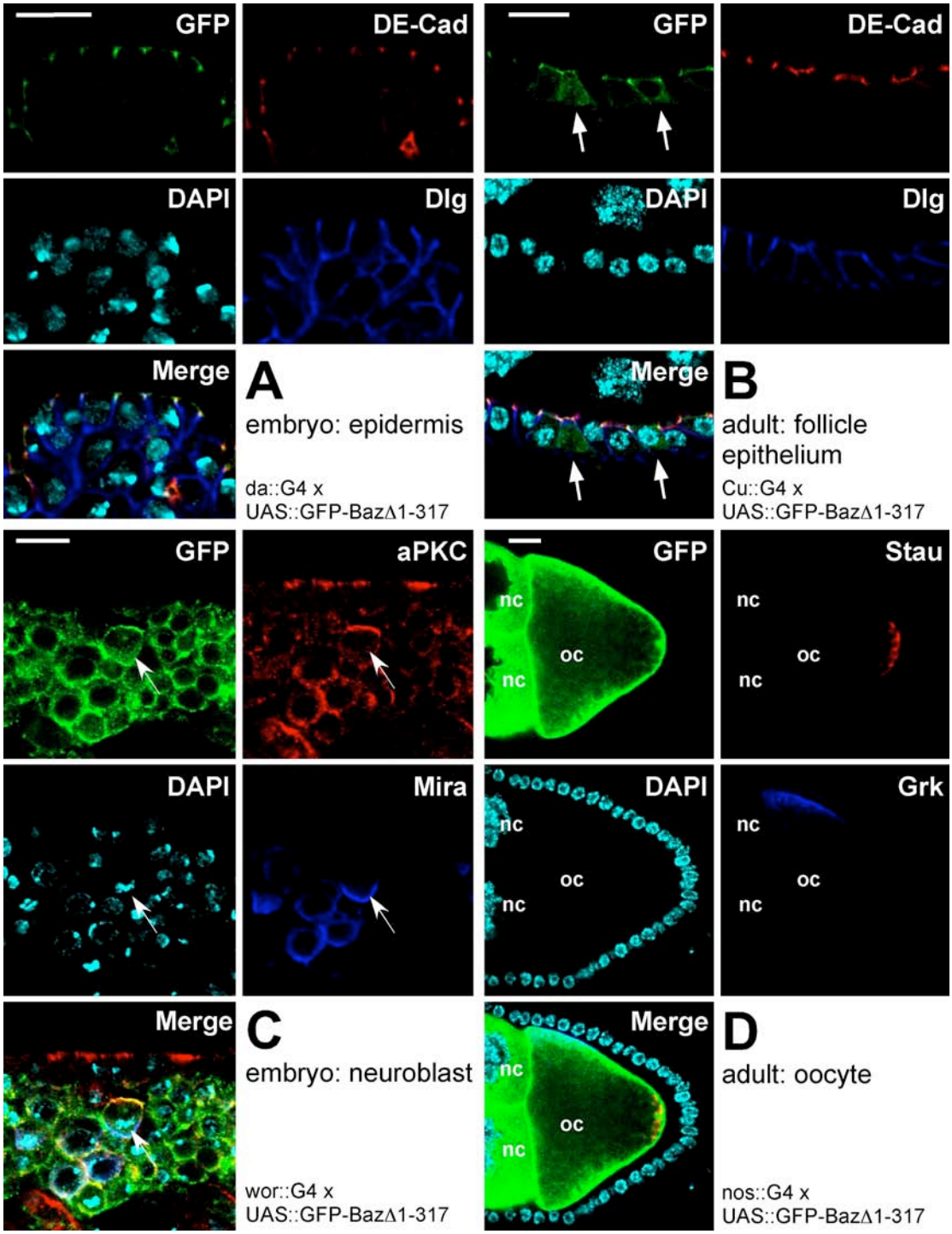
DE-Cad
DAPI
Dlg
Merge
A
embryo: epidermis
da::G4 x UAS::GFP-Baz
GFP
DE-Cad
DAPI
Dlg
Merge
B
adult: follicle epithelium
Cu::G4 x UAS::GFP-Baz
GFP
aPKC
DAPI
Mira
Merge
C
embryo: neuroblast
wor::G4 x UAS::GFP-Baz
GFP
Stau
DAPI
Grk
Merge
D
adult: oocyte
nos::G4 x UAS::GFP-Baz
nc
oc

Figure 2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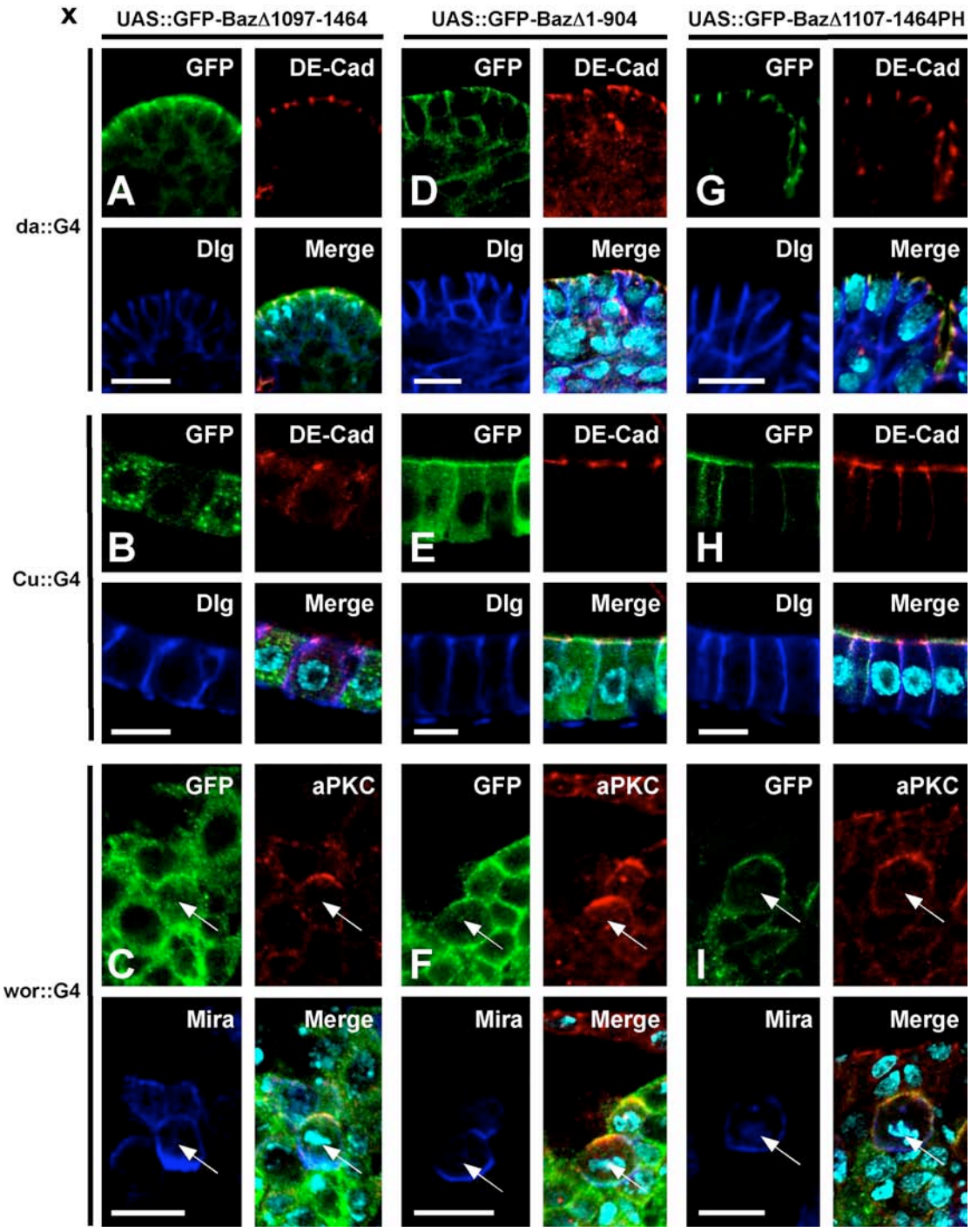
DE-Cad
DAPI
Dlg
Merge
A
embryo: epidermis
da::G4 x
UAS::GFP-BazΔ1-317
GFP
DE-Cad
DAPI
Dlg
Merge
B
adult: follicle epithelium
Cu::G4 x
UAS::GFP-BazΔ1-317
GFP
aPKC
DAPI
Mira
Merge
C
embryo: neuroblast
wor::G4 x
UAS::GFP-BazΔ1-317
GFP
Stau
DAPI
Grk
Merge
D
adult: oocyte
nos::G4 x
UAS::GFP-BazΔ1-317
nc
oc

Figure 3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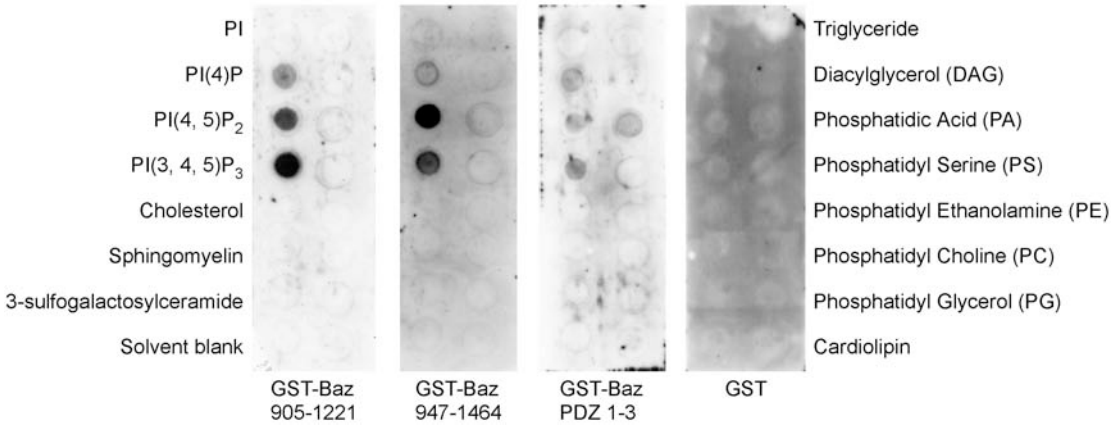
da::G4
Cu::G4
wor::G4
GFP
DE-Cad
Dlg
Merge
aPKC
Mira
A
B
C
D
E
F
G
H
I

Figure 4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PI(4, 5)P2
PI(3, 4, 5)P3
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3-sulfogalactosylceramide
Solvent blank
Triglyceride
Diacylglycerol (DAG)
Phosphatidic Acid (PA)
Phosphatidyl Serine (PS)
Phosphatidyl Ethanolamine (PE)
Phosphatidyl Choline (PC)
Phosphatidyl Glycerol (PG)
Cardiolipin
GST-Baz 905-1221
GST-Baz 947-1464
GST-Baz PDZ 1-3
GST

Figure 5

**Supplemental Material**

**Supplemental Experimental Procedures**

DNA and constructs

N-terminal deletion versions of Baz were generated by PCR from a full-length Baz cDNA clone (Krahn et al. 2009) as template using the following oligonucleotides (in 5' – 3' orientation):

BazΔ1-904-for: CACCATGTCTCCAACACTACCGGCACG

BazΔ1-904-rev: TCACACCTTGGAGGCGTGTG

BazΔ1-317-for: CACCATGGAGAGCAAGCGAAAGGAGCCC

BazΔ1-317-rev: TCACACCTTGGAGGCGTGTG

The PCR products were cloned into the pENTR vector using the pENTR Directional TOPO Cloning Kit (Invitrogen).

For generation of C-terminal deletion versions of Baz the following oligonucleotides (in 5' – 3' orientation) were used for site directed mutagenesis of wild type Baz-pEntry cDNA to introduce a premature stop codon:

BazΔ969-1464-for:

GAGACAAACTCGGGCTGAGGATCCGGAGGTCACGCCTCCAAGGTG

BazΔ969-1464-rev:

CACCTTGGAGGCGTGACCTCCGGATCCTCAGCCCGAGTTTGTCTC

BazΔ1001-1464-for: TATCAGCGGAATTAGATCTTACGCGAGGAGCGC

BazΔ1001-1464-rev: GCGCTCCTCGCGTAAGATCTAATTCCGCTGATA

BazΔ1097-1464-for: ATGGTGCAGGAGCTGTAGATGTCGGATGAGCCG

BazΔ1097-1464-rev: CGGCTCATCCGACATCTACAGCTCCTGCACCAT

BazΔ1222-1464-for: ACATCGCCGCAGCTGTGAAAGGGTGGGCGC

BazΔ1222-1464-rev: GCGCCCACCCTTTCACAGCTGCGGCGATGT

BazΔ1325-1464-for: ATGCACTCGACGAGCTGAGGATCCCAGCCAGGA

BazΔ1325-1464-rev: TCCTGGCTGGGATCCTCAGCTCGTCGAGTGCAT

BazΔ1461-1464-for:

BazΔ1461-1464-rev:

To introduce small internal deletions, the following mutagenesis primers were used:

BazΔ968-996-for: GAGACAAACTCGGGCTATCAGCGGAATAAG

BazΔ968-996-rev: CTTATTCCGCTGATAGCCCGAGTTTGTCTC

BazΔ1073-1093-for: AGGGATCAGCTGGGCCTGCAGATGTCGGAT

BazΔ1073-1093-rev: ATCCGACATCTGCAGGCCCAGCTGATCCCT

BazΔ1173-1193-for: AAGTCGTCGCGGGCCGGCGTGGTGCCAGTG

BazΔ1173-1193-rev: CACTGGCACCACGCCGGCCCGCGACGACTT

For deletion of PDZ domains, N-terminal and C-terminal fragments of Baz were amplified separately by PCR using oligonucleotides with newly introduced restriction sites and cloned into the pUAST vector. From these constructs, the region containing the respective PDZ deletion was cut out by restriction digest with Psy1 and EcoR1 and ligated into the Baz full-length pEntry construct cut with the same enzymes. The following oligonucleotides were used for PCR:

BazΔPDZ1-for1:

AAGGAAAAAAGCGGCCGCCATGAAGGTCACCGTCTGCTTCG

BazΔPDZ1-rev1: AAGGAAAAAAGCGGCCGCCTCCCTGGGCAAGGACTTGC

BazΔPDZ1-for2:

AAGGAAAAAAGCGGCCGCCCAACAGCGTGACTCTAAGGTGGC

BazΔPDZ1-rev2: GGGGTACCGCAATGTGTTCAGCACTCGGTGG

All other PDZ deletion constructs were also made using the BazΔPDZ1-for1 and BazΔPDZ1-rev2 oligonucleotides, so in the following only the respective rev1 and for2 oligonucleotides are listed:

BazΔPDZ2-rev1: CCGCTCGAGCACCTTAGAGTCACGCTGTTGG

BazΔPDZ2-for2: CCGCTCGAGAGTTCCAGTGACATTCTGGACC

BazΔPDZ3-rev1:

AAGGAAAAAAGCGGCCGCCGCCACACCAGCACTTTTCTCC

BazΔPDZ3-for2:

AAGGAAAAAAGCGGCCGCCAGTTCCAGTGACATTCTGGACCAC

BazΔPDZ1-3-rev1: CCGCTCGAGCTCCCTGGGCAAGGACTTGC

BazΔPDZ1-3-for2: CCGCTCGAGAGTTCCAGTGACATTCTGGACC

BazΔ1107-1464PHP and BazΔ1107-1464PHS were generated as follows: first, a HindIII cutting site was introduced into Baz-pEntry by site directed mutagenesis using the following oligonucleotides (in 5’ – 3’ orientation, modified nucleotides underlined) :

BazHindIIIat1107-for: CCGCGTGGTCATCAAGCTTTGCGCGCACCGCGTG

BazHindIIIat1107-for: CACGCGGTGCGCGCAAAGCTTGATGACCACGCGG

Then, the PH domain of phospholipase C δ or Steppke was amplified using the follwing primers (in 5’ – 3’ orientation) :

PLC-PH-for: AAGCTTTG GATGAGGATCTACAGGCGCT

PLC-PH-for: GGATCC CTAGATCTTGTGCAGCCCCAG

Steppke-PH-for: AAGCTTTGCCCGACAAGGAGGGCTG

Steppke-PH-rev: GGATCCTTAACTCTTGCTGAGTGCCTTTTT

Then, PCR fragments were digested with HindIII and BamHI and ligated into modified Baz-pEntryHindIII.

Using Gateway Technology (Invitrogen) all constructs were recombined into the pPGW expression vector carrying N-terminal EGFP under control of the UASp promoter.

Sequencing of mutant alleles of *baz*

Mutations in the alleles $baz^{815\text{-}8}$, $baz^{EH747}$, $baz^{XR11}$ and $baz^{Xi106}$ were identified by sequencing of PCR fragments encompassing the whole coding region of *baz* from genomic DNA of heterozygous mutant female flies balanced over FM7 using the following oligonucleotides (always in 5' – 3' orientation):

Exon 1: TTGCGAGCGAGAGCGAAGAA (forward)

ACCTCCCGTAACTCCAGAAG (reverse)

Exon 2 (5' part): CTTCCACGGCCCCCAGTCTAAT (forward)

GGGCTCCTTTCGCTTGCTCTC (reverse)

Exon 2 (3' part): GACATATCAGCAGGGTTCTCATC (forward)

CGGCCTTTTCGGTGTCGTGTA (reverse)

Exons 3 and 4: GCGAGTCGGCGGCTTCAAATGTA (forward)

GGACGTGCCGGTAGTGTTCTG (reverse)

Exon 5: CAACACGGCGCTGCTTATCG (forward)

CCAGGTCTTGCGGGGCTCTA (reverse)

Exon 6: ACCGGATGCGAGCAGTAAGTTTTT (forward)

GTGTGGGTGAGTTTGCGTGTGATG (reverse)

Exon 7 (5' part): CATGAATTTTGGCTGGCTACTTGT (forward)

CCCCTTATGCATGTGGTTTA (reverse)

Exon 7 (3’ part): TCAGCGAGGTCAACGAGGAGGTG (forward)

GGAAGTCAGCGTTGCAGTCAGTCG (reverse)

**Supplemental Figure Legends**

Supplemental Figure 1. The PDZ domains are not required for proper localization of Baz. (A) In the embryonic epidermis at stage 12, GFP-BazΔPDZ1-3 (GFP) colocalizes with DE-Cad at the ZA, but does not overlap with basolateral Dlg. (B) In the follicle epithelium at stage 10 of oogenesis, GFP-BazΔPDZ1-3 also colocalizes with DE-cad and is excluded from the basolateral membrane. (C) In embryonic metaphase NBs (arrow), GFP-BazΔPDZ1-3 colocalizes with aPKC in an apical cortical crescent opposite to the basal crescent of Mira. (D) In stage 10 oocytes, GFP-BazΔPDZ1-3 localizes to the cortex and is excluded from the posterior tip of the oocyte, marked by the presence of Stau. The anterior-dorsal region of the oocyte is marked by the Grk protein. Genotypes are indicated in the respective panels. oc, oocyte, nc, nurse cell. DNA was stained with DAPI. Scale bars = 10 μm. In (A – C) apical is up. In (D) anterior is to the left.

Supplemental Figure 2. Subcellular localization of GFP-Baz fusion proteins in S2r+ cells. Cells were cotransfected with the respective UAS::GFP-Baz constructs and actin 5C::GAL4 and stained for GFP and DAPI. For a schematic representation of the transfected constructs see Fig. 1B. Scale bars = 10 μm.

Supplemental Figure 3. Truncation of 242 aa from the C-terminus of Baz does not affect its subcellular localization. (A) In the embryonic epidermis at stage 12, GFP-BazΔ1222-1464 (GFP) colocalizes with DE-Cad at the ZA, but does not overlap with basolateral Dlg. (B) In the follicle epithelium at stage 10 of oogenesis, GFP-BazΔ1222-1464 also colocalizes with DE-cad and is excluded from the basolateral membrane. (C) In embryonic metaphase NBs (arrow), GFP-BazΔ1222-1464 colocalizes with aPKC in an apical cortical crescent opposite to

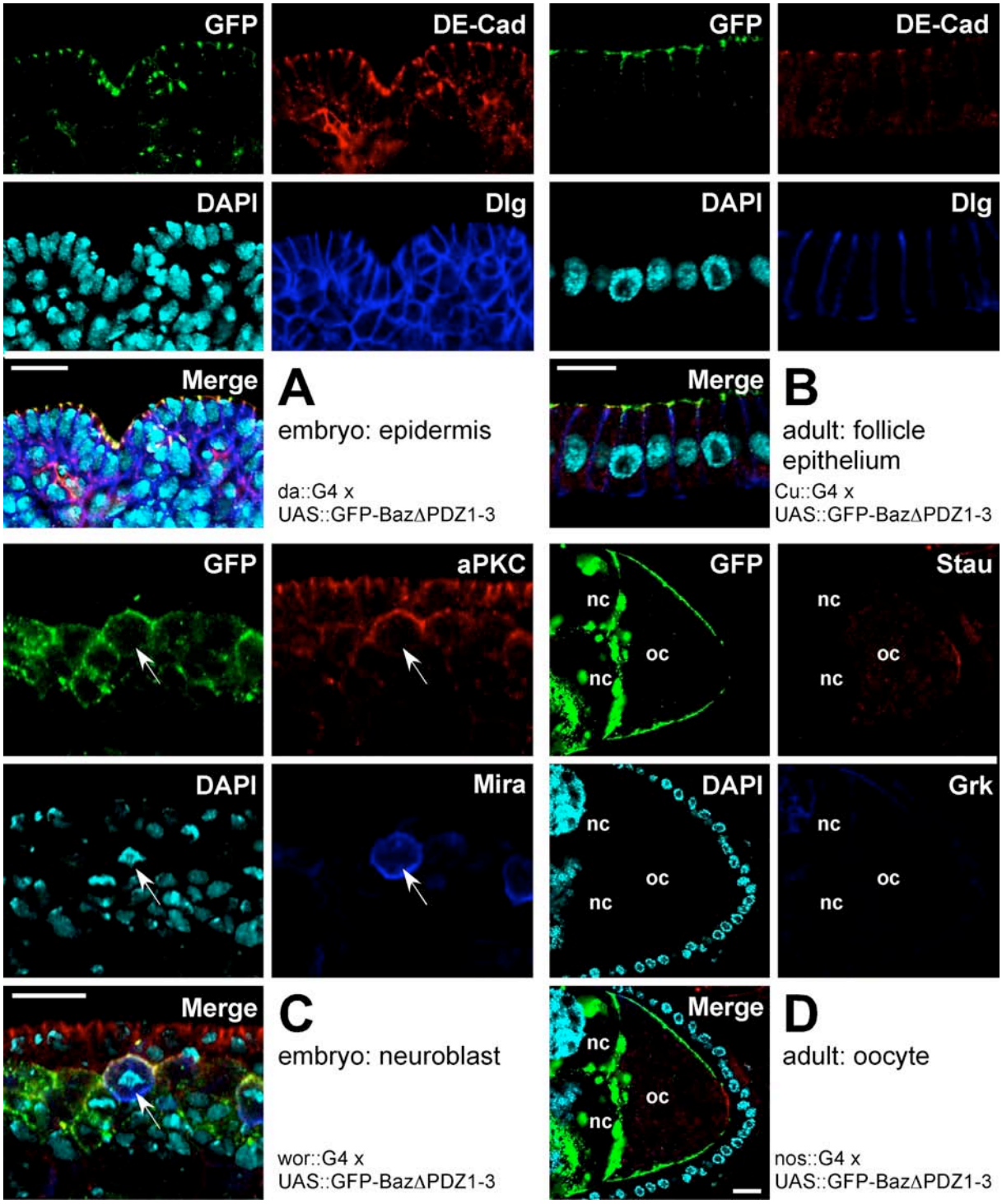
the basal crescent of Mira. (D) In stage 10 oocytes, GFP-BazΔ1222-1464 localizes to the cortex but is excluded from the posterior tip of the oocyte, marked by the presence of Stau. The anterior-dorsal region of the oocyte is marked by the Grk protein. Genotypes are indicated in the respective panels. oc, oocyte, nc, nurse cell. DNA was stained with DAPI. Scale bars = 10 μm. In (A – C) apical is up. In (D) anterior is to the left.

Supplemental Figure 4. The C-terminal 367 aa of Baz are necessary but not sufficient for membrane localization in the oocyte. (A) Deletion of aa 1097-1464 at the C-terminus of Baz leads to cytoplasmic localization of the protein in the oocyte. (B) A C-terminal fragment of Baz (aa 905-1464) also localizes to the cytoplasm and fails to associate with the oocyte membrane. (C) A chimeric GFP-Baz fusion protein in which aa 1107-1464 have been replaced by the pleckstrin homology (PH) domain of phospholipase C δ localizes uniformly to the oocyte membrane but is not excluded from the posterior pole of the oocyte, marked by Stau. The anterior-dorsal region of the oocyte is marked by the Grk protein. Genotypes are indicated to the left and to the top of the respective panels. oc, oocyte. DNA was stained with DAPI. Scale bars = 10 μm. Anterior is to the left.

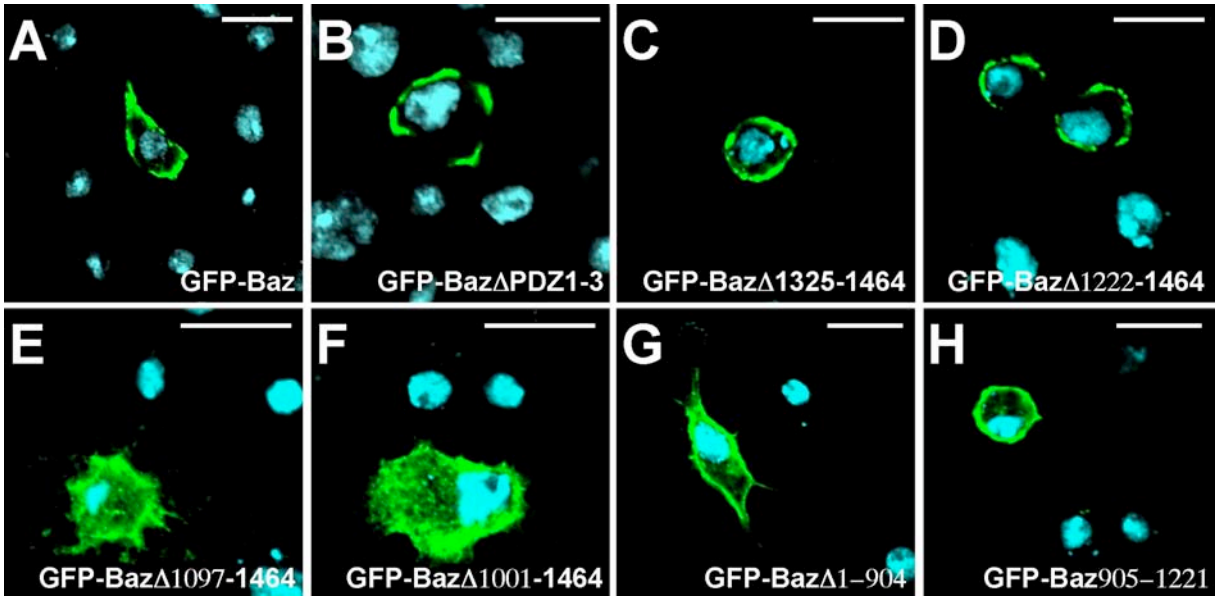
Supplemental Figure 5. Sequence conservation in the C-terminal region of Baz required for membrane binding. The sequences of Baz (Baz Dm), human PAR-3 (PAR-3 Hs), rat PAR-3 (PAR-3 Rn), chick PAR-3 (PAR-3 Gg) and zebrafish PAR-3 (PAR-3 Dr) were aligned with Megalign (DNAStar) using the Clustal V algorithm. The region between aa 898-1248 of Baz is shown. Blocks of highly conserved sequences are underlined in red. The phosphorylation sites for aPKC (S980) and PAR-1 (S1085) are marked by red arrowheads.

Supplemental Figure 6. The aPKC binding region is not required for proper localization of Baz. (A) In the embryonic epidermis at stage 12, GFP-BazΔ968-996 (GFP) colocalizes with DE-Cad at the ZA, but does not overlap with basolateral Dlg. (B) In the follicle epithelium at stage 10 of oogenesis, GFP-BazΔ968-996 also colocalizes with DE-cad and is excluded from the basolateral membrane. (C) In embryonic metaphase NBs (arrow), GFP-BazΔ968-996 colocalizes with aPKC in an apical cortical crescent opposite to the basal crescent of Mira. (D) In stage 10 oocytes, GFP-BazΔ968-996 localizes to the cortex but is excluded from the posterior tip of the oocyte, marked by the presence of Stau. The anterior-dorsal region of the oocyte is marked by the Grk protein. Genotypes are indicated in the respective panels. oc, oocyte, nc, nurse cell. DNA was stained with DAPI. Scale bars = 10 μm. In (A – C) apical is up. In (D) anterior is to the left.

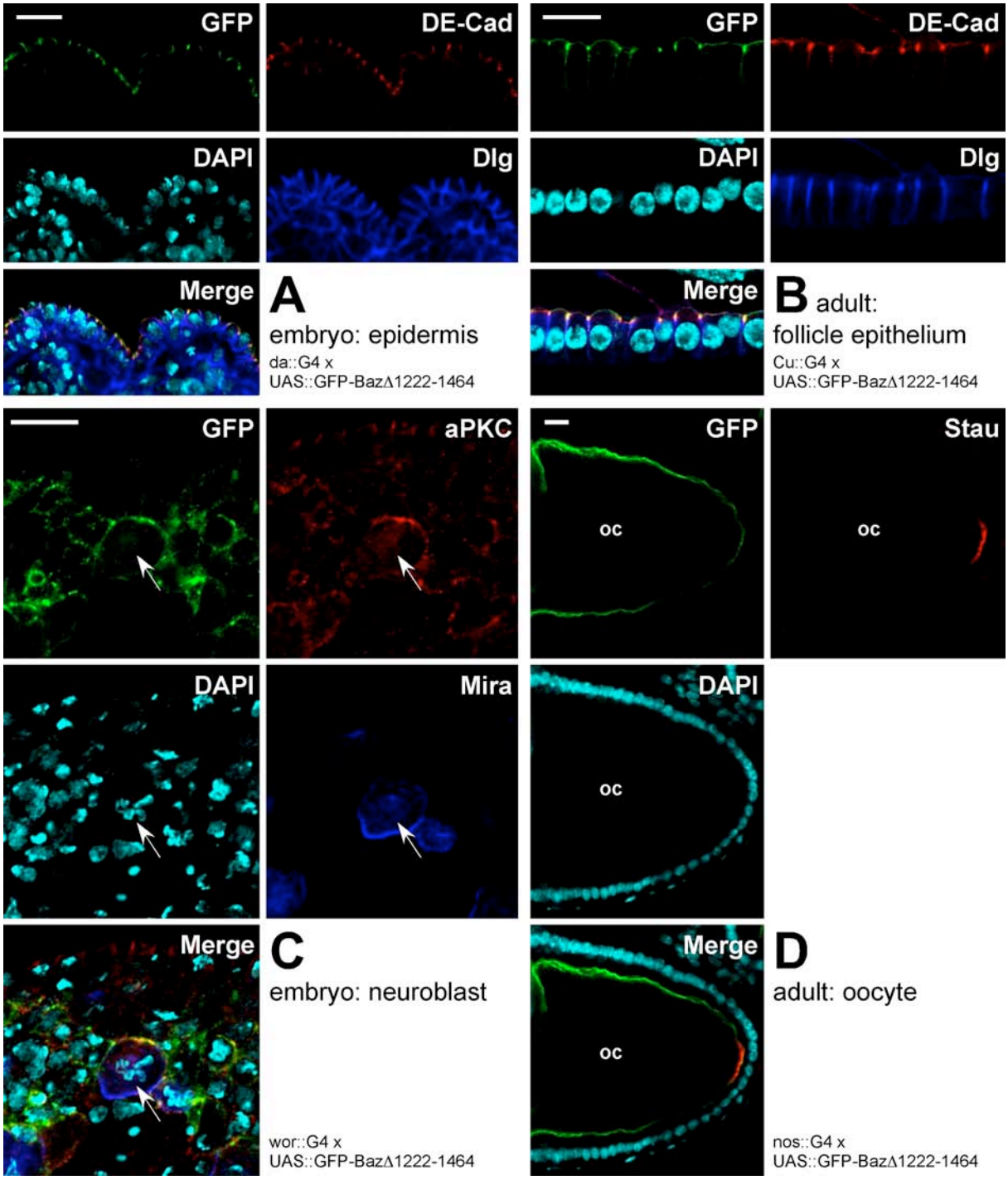
Supplemental Figure 7. The region between aa 1173-1193 is essential for membrane localization of the C-terminal phosphoinositide binding fragment of Baz. (A -D) Superficial optical sections of embryos at stage 13 expressing the GFP-Baz fusion proteins indicated on the top under control of the da::G4 driver. Embryos were stained for GFP (top row) and DE-Cad (middle row), the merged image is shown at the bottom. While in (A – C) the respective GFP-Baz fusion proteins colocalize at the plasma membrane with DE-Cad, the variant of Baz lacking aa 1173-1193 (D) is diffuse cytoplasmic. Scale bars = 10 μm.



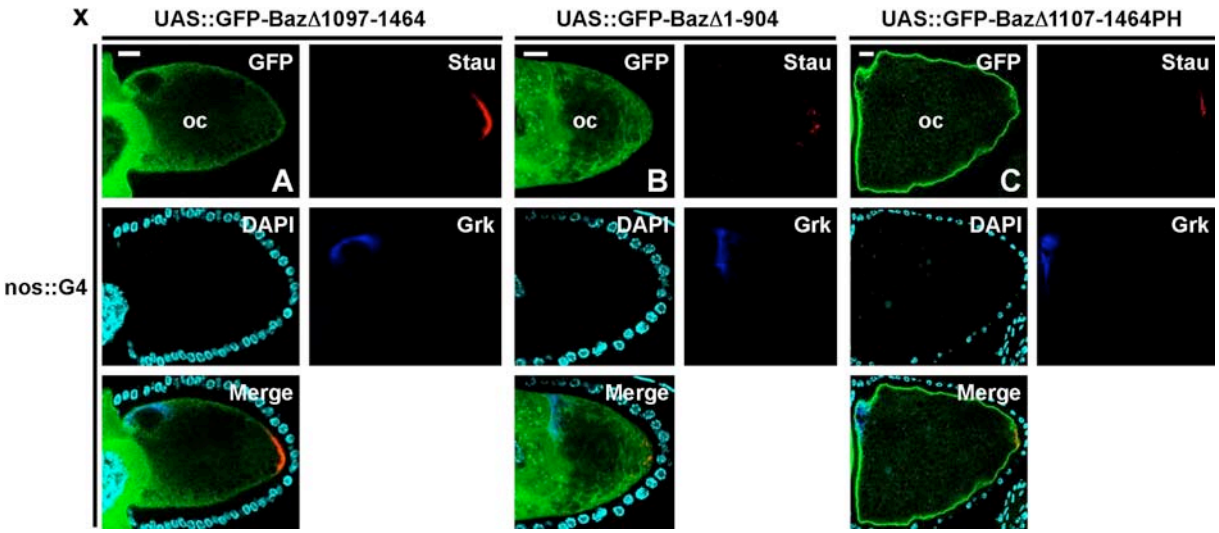

Supplemental Figure 1



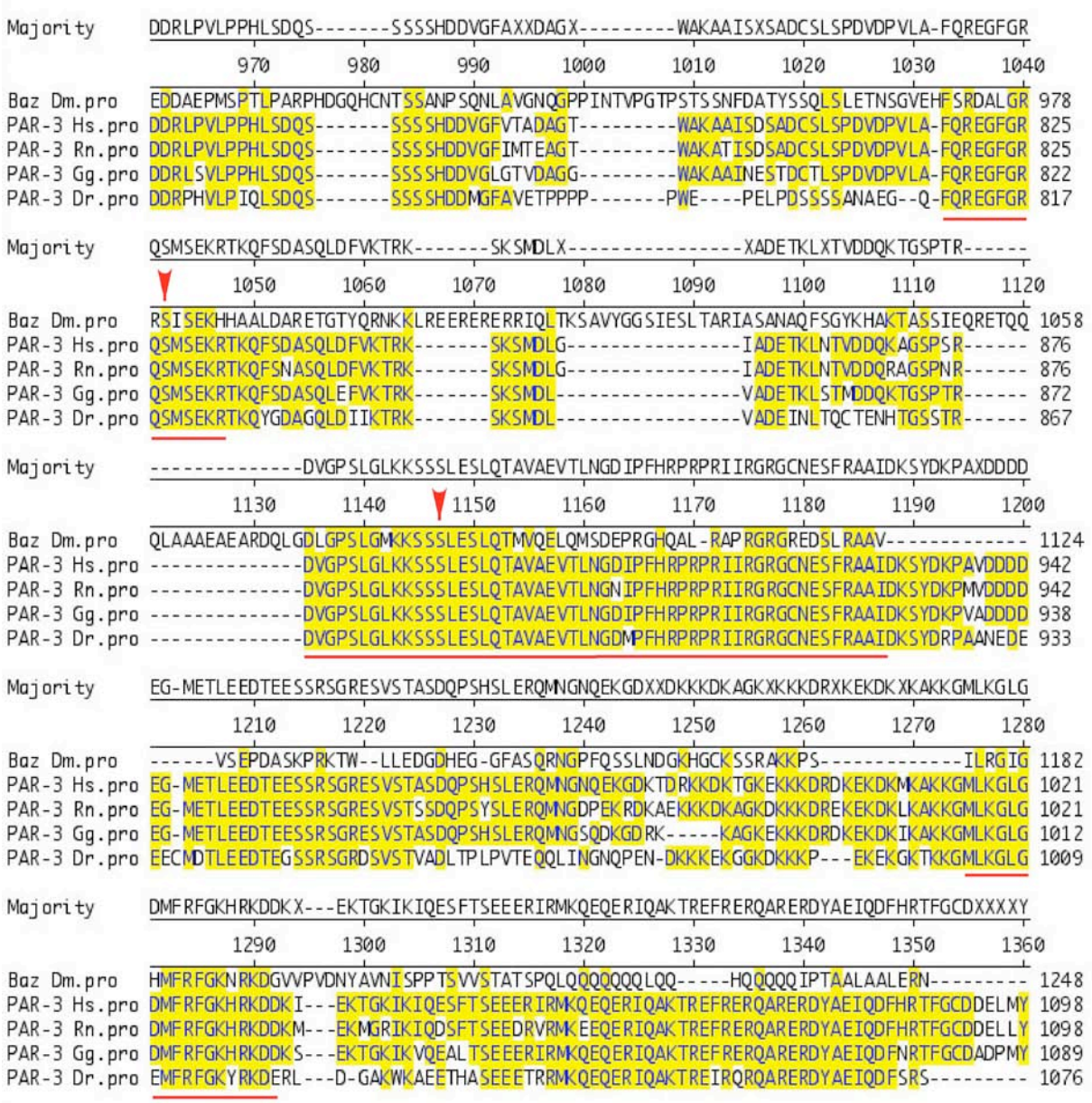

Supplemental Figure 2



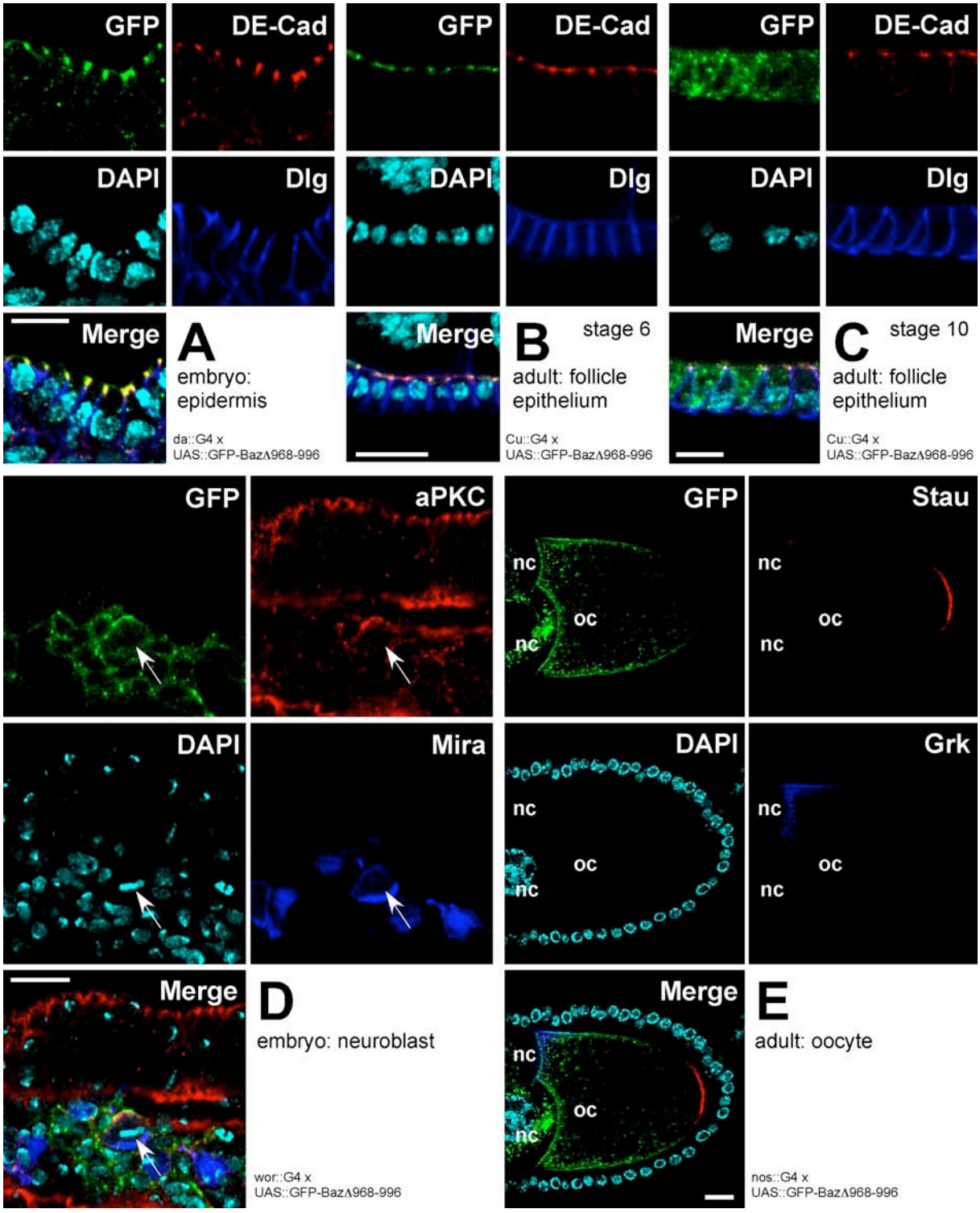

Supplemental Figure 3



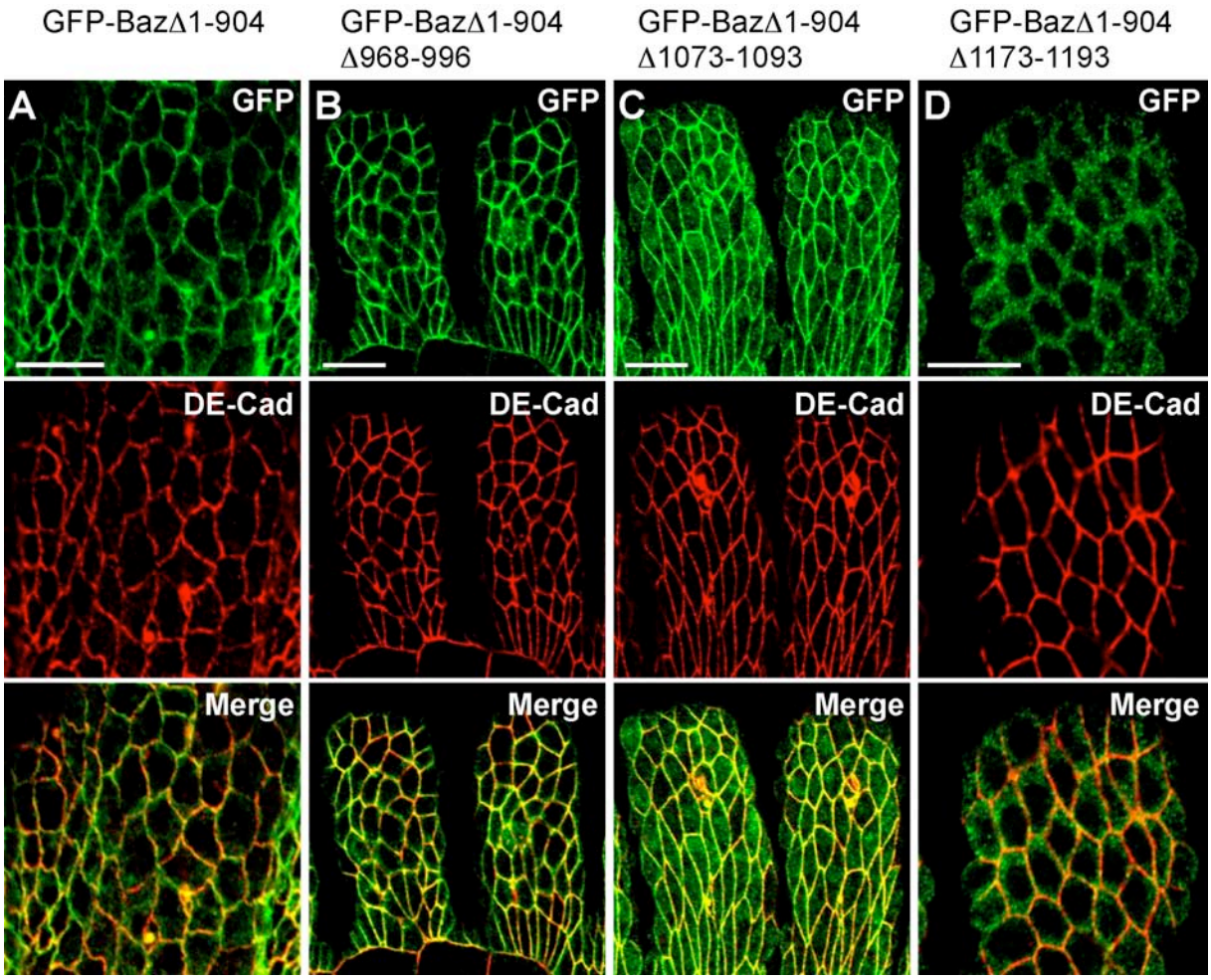

Supplemental Figure 4




Supplemental Figure 5




Supplemental Figure 6




Supplemental Figure 7

## 4.2. PP2A antagonizes phosphorylation of Bazooka by PAR-1 to control apical-basal polarity in dividing embryonic neuroblasts

In this part I investigated the interaction between the Bazooka protein and one particular potential interaction partner, protein phosphatase 2A (PP2A), which was found in a yeast-two-hybrid screen in the lab before.

After verification of the interaction by co-immunoprecipitation experiments, I showed that of the three described phosphorylation sites, only one is dephosphorylated by PP2A in vivo.

Furthermore, the effect of PP2A phosphatase activity knock down or the overexpression of the complementary kinase activity (PAR-1) on the apical-basal polarity in metaphase NBs is was investigated.

**Michael P. Krahn**, Diane Egger-Adam and Andreas Wodarz

Author contributions to the work:

| | |
|---|---|
| **Michael P. Krahn**: | All experiments, besides*<br>writing of the manuscript |
| Diane Egger-Adam: | *Initial Yeast-Two-Hybrid-Screen for new interaction partners of<br>Baz, in which PP2A was found |
| Andreas Wodarz: | Editing of the manuscript |

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# PP2A antagonizes phosphorylation of Bazooka by PAR-1 to control apical-basal polarity in dividing embryonic neuroblasts

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**Abstract**

Bazooka/Par-3 (Baz) is a key regulator of cell polarity in epithelial cells and neuroblasts (NBs). Phosphorylation of Baz by PAR-1 and aPKC is required for its function in epithelia, but little is known about the dephosphorylation mechanisms that antagonize the activities of these kinases or about the relevance of Baz phosphorylation for NB polarity. We found that protein phosphatase 2A (PP2A) binds to Baz via its structural A subunit. By using phospho-specific antibodies, we show that PP2A dephosphorylates Baz at the conserved serine residue 1085 and thereby antagonizes the kinase activity of PAR-1. Loss of PP2A function leads to complete reversal of polarity in NBs, giving rise to an “upside down” polarity phenotype. Overexpression of PAR-1 or Baz, or mutation of 14-3-3 proteins that bind phosphorylated Baz, causes essentially the same phenotype, indicating that the balance of PAR-1 and PP2A effects on Baz phosphorylation determines NB polarity.

## Introduction

The generation of cell fate diversity in developing and adult organisms depends on the asymmetric division of stem cells. One of the best model systems to study this process are the embryonic neuroblasts (NBs) in the fruit fly *Drosophila*. Upon unequal cytokinesis, the NB gives rise to a ganglion mother cell (GMC) which divides only once more and produces two neurons or glia cells, and another NB that continues to divide asymmetrically (Wodarz, 2005; Knoblich, 2008; Zhong and Chia, 2008). A crucial prerequisite for asymmetric cell division is the polarization of the stem cell. NB polarity is controlled by a group of genes encoding cortical proteins which function in a hierarchy, leading to the asymmetric localization of the cell fate determinants Prospero, Brain Tumor (Brat) and Numb (Wodarz, 2005; Knoblich, 2008; Zhong and Chia, 2008).

The PDZ domain protein Baz, the *Drosophila* homolog of *C. elegans* and vertebrate PAR-3, is among the first proteins that localize to the apical NB cortex where it recruits PAR-6 and aPKC to form the Baz/PAR-6/aPKC complex (Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999; Wodarz et al., 2000; Petronczki and Knoblich, 2001). Together with the proteins Discs Large (Dlg), Lethal giant larvae (Lgl) and Scribble (Scrib), the Baz/PAR-6/aPKC complex is essential for the localization of cell fate determinants and their adaptor proteins Miranda (Mira) and Partner of Numb (Pon) to the basal cortex of mitotic NBs (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Ohshiro et al., 2000; Peng et al., 2000; Albertson and Doe, 2003). The assembly and activity of the Baz/PAR-6/aPKC complex is controlled by a phosphorylation cascade triggered by

activation of the mitotic kinase Aurora A, which leads to phosphorylation of PAR-6 and activation of aPKC (Wirtz-Peitz et al., 2008). aPKC in turn phosphorylates and inactivates Lgl at the apical cortex (Betschinger et al., 2003). Phosphorylation of Lgl leads to dissociation of Lgl from PAR-6 and aPKC and to its exchange for Baz. The Baz/PAR-6/aPKC complex finally phosphorylates Numb and prevents its localization to the apical NB cortex (Wirtz-Peitz et al., 2008). These findings clearly show that modification of polarity proteins by phosphorylation is an important mechanism to regulate their function in asymmetric cell division.

Baz itself is also a phosphoprotein and some phosphorylation sites relevant for its function have been identified. Phosphorylation of S151 and S1085 by PAR-1 is crucial for Baz function and localization in cells of the follicle epithelium and in the oocyte (Benton and St Johnston, 2003). In rat PAR-3, phosphorylation of S827, corresponding to S980 in Baz, by aPKC results in decreased stability of the aPKC-PAR-3 interaction (Nagai-Tamai et al., 2002). Overexpression of a mutant version of PAR-3 that cannot be phosphorylated at this site (PAR-3 S827A) in MDCK cells leads to polarity defects similar to those observed upon overexpression of a dominant-negative form of aPKC (Suzuki et al., 2001; Nagai-Tamai et al., 2002).

In contrast, up to now little is known about the mechanisms of Baz or PAR-3 dephosphorylation counteracting the activities of PAR-1 and aPKC kinases. Protein phosphatase 1 (PP1) has recently been shown to bind mouse PAR-3 and to dephosphorylate its serine residues S144 and S824, corresponding to S151 and S980 of Baz (Traweger et al., 2008). A function for protein phosphatase 2A (PP2A) as an antagonist of PAR-1 in photoreceptor cells of

the *Drosophila* pupal retina was recently described (Nam et al., 2007). In this system, overexpression of PAR-1 or reduction of PP2A activity led to mislocalization of Baz and disturbed cell polarity.

PP2A is a ubiquitously expressed serine-threonine-specific phosphatase which has crucial functions in various cellular processes such as cell cycle control, cytoskeleton reorganization and cell polarity (Janssens and Goris, 2001). PP2A is a heterotrimeric enzyme complex consisting of a catalytic (PP2A-C) subunit, which is only stable if tightly bound to a structural subunit of 65kD (PP2A-A). This core complex recruits one out of several regulatory subunits (PP2A-B) which provide substrate specificity (Janssens and Goris, 2001). Whereas in vertebrates two different A subunits and various regulatory B subunits are expressed, in *Drosophila* the A subunit is encoded by a single gene and only four genes for regulatory B subunits are annotated: Twins (Tws), Widerborst (Wdb), B56-1 and PR72.

In this study we show that a heterotrimeric complex of PP2A-A, PP2A-C(Mts) and Tws binds to Baz and dephosphorylates Baz at the conserved serine residue 1085. Furthermore, impaired dephosphorylation of this site caused by lack of PP2A activity induces a dramatic phenotype in late stage embryonic NBs characterized by a total reversal of apical-basal cell polarity. This finding can be phenocopied either by overexpression of PAR-1 or Baz. We furthermore show that the *Drosophila* homologs of PAR-5, 14-3-3ε and 14-3-3ζ (Leonardo), function in the same pathway to control apical-basal NB polarity.

**Results**

PP2A is found in a complex with Baz but not with aPKC or PAR-6

In a yeast two-hybrid screen for interaction partners of the N-terminal region of Baz (aa 1-318), we isolated a clone corresponding to aa 399-590 of the structural A subunit of PP2A (PP2A-A) that specifically interacted with the Baz bait (Figure S1). In an independent experimental approach, we performed coimmunoprecipitation experiments from embryonic extracts using an antibody directed against Baz. A 39 kD band coimmunoprecipitating with Baz was analyzed by mass spectrometry and three peptides matched Mts, the only catalytic subunit of PP2A annotated in the *Drosophila* genome (Figure S1).

In order to test whether PP2A is able to bind Baz in vivo, we performed coimmunoprecipitation experiments from embryonic lysates. To that aim, we generated polyclonal peptide antisera directed against PP2A-A. These antisera recognize a single band of 65 kD in Western blots, which corresponds to the predicted size of PP2A-A (Figure 1A). A small proportion of PP2A-A and Mts coimmunoprecipitated with Baz (Figure 1A). Vice versa, GFP-Baz was present in immunoprecipitates pulled down with the anti PP2A-A antibody (Figure 1B). In contrast, neither aPKC nor PAR-6 were found in a complex with PP2A (Figure 1C, D).

To clarify which of the four regulatory B-subunits encoded in the *Drosophila* genome were associated with the PP2A-Baz complex, HA-tagged versions of all four B-subunits were coexpressed with GFP-Baz in S2 cells and tested for coimmunoprecipitation with GFP-Baz. Both the Tws (Figure 1E) and the B56-1 (Figure 1F) regulatory subunits were found in a complex with GFP-Baz, whereas no interaction was found with Wdb (Figure 1G) or PR72 (Figure 1H).

Inhibition of PP2A activity in S2 cells results in increased phosphorylation of Baz at serine 1085

To test whether the association of Baz with PP2A controls the phosphorylation of one of the three previously described phosphorylation sites of Baz, we raised phospho-specific peptide antibodies against each of these phosphorylated serine residues (pS151, pS980, pS1085). S2 cells overexpressing GFP-Baz were treated for 4h with either DMSO as negative control, with okadaic acid (5 nM) or with cantharidin (0.5 µM). At these inhibitor concentrations, the inhibition of PP2A is more than tenfold higher than the inhibition of PP1, another major cellular protein phosphatase that can be inhibited with these substances (Barford, 1996). Subsequently, cells were lysed, GFP-Baz was immunoprecipitated and the precipitates were subjected to Western blot with the phospho-specific antibodies (Figure 2B - D). Whereas GFP-Baz from DMSO treated cells was only weakly phosphorylated at any of the three investigated sites, treatment with the phosphatase inhibitors resulted in a strong increase of the band intensity with all three phospho-specific antibodies (Figure 2B - D). In contrast, the total amount of Baz protein was not affected (Figure 2A). Treatment of the cells with 100µM cantharidin, a 10-fold excess over the $EC_{50}$ of PP1, did not lead to further increase of phosphorylation at any of the three serines (Figure 2B – D). However, a distinct shift in the mobility of the Baz band was detectable, indicating that PP1 or other phosphatases affected by this high concentration of the inhibitor may dephosphorylate other, yet undescribed phosphorylation sites of Baz.

In contrast to the three phosphorylation sites in Baz, phosphorylation of aPKC at T422 was not affected by the low concentrations of cantharidin or okadaic

acid that predominantly inhibit PP2A (Figure 2G). Increased phosphorylation of T422 was only observed after inhibition of PP1 by higher concentrations of cantharidin (Figure 2G).

While these inhibitor experiments pointed to PP2A as the phosphatase that specifically dephosphorylated the three serine residues of Baz, the specificity of the inhibitors is not high enough to exclude the involvement of other phosphatases, such as PP4 or PP6. We therefore knocked down PP2A by application of double-stranded RNA in S2 cells, which has been shown to work efficiently for PP2A subunits (Silverstein et al., 2002; Sathyanarayanan et al., 2004). This approach also allowed us to clarify which one of the different regulatory B subunits is involved in the dephosphorylation of Baz.

Knock down of PP2A-A, Mts and Tws resulted in strongly elevated phosphorylation of S1085 (Figure 2K), whereas phosphorylation of S151 and S980 remained unaffected (Figure 2I, J). In the absence of phosphatase inhibitors only a smaller form of Baz (110 kD) was detectable with the anti BazpS151 antibody (Figure 2I, cf. Figure 2B). Knock down of Wdb, B56-1 and PR72 did not lead to elevated phosphorylation of S1085 (Figure 2K), indicating that Tws is the only regulatory B subunit of PP2A that functions in a complex with the core dimer of PP2A-A and Mts to dephosphorylate Baz at S1085. PP2A-A and Mts were mutually required to stabilize each other and the B subunits Tws and Wdb, whereas the knock-down of Wdb or Tws did not affect the stability of PP2A-A or Mts (Figure 2L – O) (Silverstein et al., 2002).

Apical-basal polarity is totally reversed in a fraction of PP2A deficient metaphase-NBs

In order to investigate the consequences of PP2A depletion in embryonic NBs, we examined embryos homozygous mutant for *PP2A-29B*$^{GE16781}$, a predicted null allele for the PP2A-A subunit which carries a P-element insertion 38 bp 3' of the start-codon and is embryonic lethal. The lethality of *PP2A-29B*$^{GE16781}$ was fully rescued by ubiquitous expression of a UAS::PP2A-29B transgene (data not shown). In NBs at early stages of neurogenesis (stage 9-11) we did not observe any abnormalities regarding spindle orientation or localization of polarity proteins (Figure 3B). However, in late neurogenesis (stage 13-15), a significant percentage of *PP2A-29B*$^{GE16781}$ mutant NBs showed either a spindle orientation parallel to the plane of the neuroectodermal epithelium (27%, n=99; Figure 3E, J) or a total reversal of apical-basal cell polarity (22%; Figure 3D, J). Very few NBs with oblique spindle orientation were found in *PP2A-29B*$^{GE16781}$ mutant embryos (Figure 3J). In the NBs with reversed polarity, Baz, which localizes to the apical cortex in wild type (Figure 3A, C) formed basal crescents (Figure 3D), while Mira, which localizes to the basal cortex in wild type (Figure 3A, C), formed apical crescents (Figure 3D). Analysis of other apically (Figure S2A – H) and basally (Figure S2I – N) localized proteins confirmed that cortical polarity was completely reversed in *PP2A* mutant embryos. To verify that this phenotype was due to decreased activity of PP2A, we analyzed embryos homozygous mutant for *mts*$^{XE2258}$ (Figure 3F) (Wassarman et al., 1996) and *tws*$^{60}$ (Figure 3G) (Uemura et al., 1993) and found essentially the same abnormalities. To analyze the functional requirement for PP2A in NB polarity by an additional approach, we expressed a dominant negative version of Mts (UAS::dnMts) (Hannus et al., 2002) in a NB specific pattern using wor::GAL4 or in a

ubiquitous pattern using tubulin::GAL4 or mat67::GAL4. In all of these cases we identified “upside down” NBs at late stages of neurogenesis at a frequency comparable to embryos homozygous for mutations in *PP2A-A*, *mts* or *tws* (Figure 3I). No polarity defects were observed in NBs at earlier stages of neurogenesis (Figure 3H), although the UAS::dnMts was expressed from the beginning of neurogenesis when the maternal tubulin::GAL4 or mat67::GAL4 driver lines were used. We did not detect any *PP2A-29B*$^{GE16781}$ mutant ana- or telophase NBs showing reversed polarity, which is probably due to an arrest in mitosis upon loss of PP2A activity (Chen et al., 2007).

The NB polarity phenotype of *PP2A* mutants can be phenocopied by overexpression of PAR-1

PAR-1 can phosphorylate Baz at relevant serine residues S151 and S1085 (Benton and St Johnston, 2003) and acts as an antagonist of PP2A with respect to cell polarity and Baz localization in pupal photoreceptor cells (Nam et al., 2007). In NBs, GFP-tagged PAR-1 localized predominantly to the basal cortex (Figure S3A). To test whether PAR-1 antagonizes PP2A also in the control of NB polarity, we overexpressed PAR-1 in NBs using the UAS-GAL4 system. The consequences on NB polarity at metaphase were essentially the same as in *PP2A* mutants and upon overexpression of dominant negative Mts (Figure 3L; Table 1). Again, polarity defects were only observed in older NBs from stage 13 onwards. The reversion of NB polarity was dependent on the kinase activity of PAR-1, as overexpression of a kinase-dead version of PAR-1 (PAR-1T408A) did not result in NB polarity defects (Figure 3O; Table 1). In contrast to the situation in *PP2A* mutants, overexpression of PAR-1 did not result in mitotic arrest at metaphase. Consequently, we did observe a

significant number of ana- and telophase NBs that budded off the GMC to the apical side (Figure 3M), confirming our hypothesis that these NBs are entirely upside-down. Besides NBs dividing with an upside down polarity we also observed an increased number of telophase NBs dividing in an orientation parallel to the plane of the neuroectodermal epithelium (Figure 3N).

Moderately elevated level of Baz results in reversed apical-basal NB polarity

We next addressed the question of whether changes in Baz phosphorylation are responsible for the reversal of NB polarity upon loss of PP2A function or overexpression of PAR-1. Using different driver lines (Wor::GAL4, Pros::GAL4, asense::GAL4) and different temperatures, we determined that the effects of Baz overexpression were dosage dependent. Weak overexpression of wild type Baz, either untagged or N-terminally tagged with GFP, led to complete reversal of NB polarity (Figure 3P) in a significant fraction of NBs from stage 13 onwards (23.3%, Table 1). Similar to PP2A knockdown or PAR-1 overexpression, the number of dividing NBs showing a spindle orientation rotated by 90° was also increased (20.8%, Table 1). Furthermore, we frequently observed NBs with reversed polarity in ana- and telophase, budding off the GMC to the apical side, similar to the phenotype upon PAR-1 overexpression (Table 1). In order to visualize this event and to further elucidate if the reversed NB polarity was immediately established or was caused by relocalization of initially correctly targeted apical and basal proteins, we performed live imaging of embryos co-overexpressing wild type Baz and Pon-GFP (Lu et al., 1999) (Movies S2 and S3). In wild type embryos, Pon-GFP accumulated at the basal cortex and exclusively segregated into the basally localized GMC (Movie S1). By contrast, upon overexpression of Baz

we frequently observed asymmetric divisions in parallel to the plane of the overlying epithelium (Movie S2) and also asymmetric divisions in which the GMC budded off to the apical side (Movie S3). In the latter case, Pon-GFP was directly targeted to the apical cortex without prior accumulation at the basal cortex (Movie S3).

Our observations bring up the question of whether the reversal of cortical apical-basal NB polarity was due to a mistargeting of apical and basal protein complexes without affecting the intrinsic asymmetry of the mitotic spindle, or whether the whole NB, including the mitotic spindle, was upside down. To investigate this, we stained centrosomes with γ-tubulin (Figure S4) and Cnn (data not shown). In wild type, the apical centrosome of metaphase and anaphase NBs was significantly larger than the basal one (Figure S4A, B) (Kaltschmidt et al., 2000). In NBs showing reversed apical-basal polarity upon overexpression of Baz, the basal centrosome was larger than the apical centrosome (Figure S4C). In general, the centrosome close to the Baz crescent was larger than the centrosome opposite to the Baz crescent, irrespective of the orientation of the NB relative to the overlying epithelium (Figure S4C, D). From these observations we conclude that the affected NBs do not only exhibit reversed apical-basal polarity of the cytocortex but are entirely upside down.

Next we investigated if the phosphorylation of serine 1085 is crucial for the polarity reversal in NBs. Overexpression of Baz in which serine 1085 was exchanged for glutamate (BazS1085E), mimicking a constitutively phosphorylated protein, showed similar effects as overexpression of wild type Baz (20.4% reversed polarity and 25.7% spindle rotated 90°, Table 1) without

further enhancement of the phenotype, consistent with our observation that wild type Baz gets phosphorylated on S1085 upon overexpression (Figure S5). In contrast, overexpression at the same level (Figure S6) of nonphosphorylatable Baz in which serine 1085 was exchanged for alanine (BazS1085A) caused polarity reversal in only 4,1% of NBs and spindle misorientation in 14,6% of NBs (Table 1). Overexpression of a version of Baz in which both S151 and S1085 were mutated to alanine (BazS151AS1085A) did not cause any significant increase of NBs with abnormal polarity compared to wild type (Table 1), suggesting that S151 contributes to a minor extent to the polarity defects observed upon overexpression of Baz.

14-3-3 proteins interact with Baz in the control of neuroblast polarity

In *Drosophila*, two homologues of PAR-5 exist: 14-3-3ε and 14-3-3ζ (Leonardo, Leo). Both have been shown to interact with Baz posphorylated by PAR-1 and thereby exclude Baz from the basolateral membrane in the follicle cell epithelium (Benton and St Johnston, 2003). We have confirmed the phosphorylation-dependent interaction of 14-3-3ε with S1085 of Baz by coimmunoprecipitation experiments in transfected S2 cells (Figure S7). Interestingly, the binding of 14-3-3ε to Baz was inversely correlated with binding of aPKC to Baz (Figure S7), suggesting that phosphorylation of S1085 regulates the binding of Baz to aPKC.

In embryos mutant for a loss-of-function and a hypomorphic allele of *14-3-3ζ* (*14-3-3leo*$^{12BL}$ and *14-3-3leo*$^{P1188}$), we detected the same phenotype of reversed apical-basal NB polarity as in *PP2A* mutants and upon PAR-1 overexpression (Figure 3Q, Table 1 and data not shown). In zygotic mutants

of a null allele of *14-3-3ε*, *14-3-3ε$^{j2B10}$*, only a few (6.5%, Table 1) embryonic NBs showed reversed polarity, which could be due to the fact that the maternal component is stronger and longer lasting than that of *14-3-3ζ.* Indeed, zygotic mutant *14-3-3ε$^{j2B10}$* animals survive until late larval stages.

Based on these findings we speculated that like in the follicle epithelium, 14-3-3 proteins may be required to exclude Baz from the basal cortex in NBs and thus to ensure proper NB polarity. To test our hypothesis, we co-overexpressed wild type Baz and 14-3-3ζ or 14-3-3ε in NBs. Indeed, the percentage of NBs showing a reversed polarity was strongly decreased compared to overexpression of Baz alone (Table 1).

## Discussion

Apical-basal polarity of NBs is controlled by a relatively small number of proteins which assemble into protein complexes localized to the NB cortex in an asymmetric fashion (Wodarz, 2005; Knoblich, 2008). These cortical proteins interact with each other in a functional hierarchy. At the top of the hierarchy is Baz, because it can localize to the apical NB cortex in loss-of-function mutants for any of the other factors, including PAR-6, aPKC, Insc, Pins and others (Schober et al., 1999; Wodarz et al., 1999; Yu et al., 2000; Rolls et al., 2003); Kim et al., submitted).

Here we have shown that Baz gets frequently mislocalized to the basal NB cortex when it is moderately overexpressed or when it is excessively phosphorylated at S1085, either by overexpression of PAR-1 or by loss-of-function of PP2A. We expect that similar antagonistic activities of kinases and

phosphatases regulate the phosphorylation state of additional sites of Baz/PAR-3 that are relevant in different cellular contexts. Loss-of-function of *14-3-3ζ* and to a lesser extent of *14-3-3ε* causes mislocalization of endogenous Baz in NBs, whereas overexpression of 14-3-3ζ and 14-3-3ε suppresses the mislocalization of overexpressed Baz. We therefore suggest that the ratio of Baz phosphorylated at S1085 to the amount of available 14-3-3 determines whether Baz gets mislocalized to the basal cortex. In this model, the 14-3-3 proteins function as a buffer to inactivate mislocalized, phosphorylated Baz. This inactivation could be explained by the inhibition of aPKC binding to Baz upon association of 14-3-3 with Baz. If the amount of overexpressed Baz exceeds the buffering capacity of 14-3-3, this would lead to the formation of active Baz/aPKC complexes at the basal cortex. These basally localized, active Baz/aPKC complexes may in turn affect the localization of PAR-1. The mammalian aPKC homolog PKCζ can phosphorylate PAR-1 at a conserved serine residue and this phosphorylation causes a strong reduction of PAR-1 kinase activity and the release of PAR-1 from the plasma membrane (Hurov et al., 2004). If the same was true in *Drosophila*, it would explain the total reversal of NB polarity, because the now basally localized aPKC would phosphorylate PAR-1, which would cause its release from the membrane and the establishment of a new apical cortical domain at the previously basal cortex.

PAR-1, 14-3-3 proteins and PP2A are strongly expressed during oogenesis and maternal contributions may account for difficulties identifying requirements during early embryogenesis. On the other hand, eliminating maternal expression of these genes results in phenotypes too severe to allow

the study of neurogenesis (Wassarman et al., 1996; Shulman et al., 2000; Benton et al., 2002). However, overexpression of a dominant-negative form of Mts from early neurogenesis onwards also caused polarity reversal only in late stage NBs. While this experiment does not exclude the possibility that the late onset of polarity reversal in NBs is due to the perdurance of the maternal gene products, it points to a fundamental difference in the mechanism of how NB polarity is controlled immediately after delamination as opposed to subsequent asymmetric divisions. The majority of late stage NBs showing polarity reversal were not in direct contact with the overlying epithelium and thus may rely exclusively on intrinsic polarity cues, in contrast to NBs that have just delaminated and maintain contact to the overlying epithelium. Late stage NBs lacking contact to the overlying epithelium show a higher variability of spindle orientation as compared to early stage NBs in close contact to the epithelium (Siegrist and Doe, 2006). Thus, late stage NBs may be particularly sensitive to changes in the phosphorylation state and general activity level of Baz, because they rely on Baz as the main cue for orienting their polarity axis.

It is interesting to note that mutations uncoupling spindle orientation from the localization of cell fate determinants commonly show fully random spindle orientation, including a variety of oblique orientations (Izumi et al., 2006; Siller and Doe, 2009). In contrast, hyperphosphorylation of Baz at S1085 resulted very rarely in oblique orientations and spindles were always aligned with the asymmetric crescents of cell fate determinants. Although we currently do not have a good explanation for why there is a strong bias for either total reversal of polarity or misorientation of the spindle by 90°, our findings point to the

existence of a spatial cue functioning upstream of Baz that defines a polarity axis perpendicular to the plane of the epithelium.

## Experimental Procedures

### Antibodies

Phospho-specific antibodies against Baz phosphorylated at serine 151, 980 and 1085 were raised by injection of the phosphorylated peptides CLMVRRSpSDPNLL (pS151), CDALGRRpSISEKH (pS980) and CGMKKSSpSLESLQ (pS1085) into rabbits and subsequent affinity purification against the phosphorylated peptide.

Peptide antibodies directed against PP2A-A (PP2A-29B) were raised by injection of the peptides AASDKSVDDSLYPIAC (aa 2-16) and PYVRDLVSDPNPHVKC (aa 330-344) into rabbits (Eurogentec, Seraing, Belgium).

### Immunoprecipitation and Western blotting

For immunoprecipitations, wild type embryos from an overnight collection were dechorionated and lysed in lysis buffer (1% Triton X-100, 150mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 25mM Hepes pH 7.44) supplemented with protease inhibitors. S2 cells were lysed in the same buffer. After centrifugation, 2 μl of rat anti Baz (Wodarz et al., 1999), 2 µl of rabbit anti PP2A-A, 2µl of rabbit anti PKCζ C20 (Santa Cruz Biotechnology, Inc.), 2 μl of guinea-pig anti PAR-6, 2 μl of rabbit anti-GFP (Molecular Probes #A11122), or 2 µl of the corresponding preimmune serum were added to cell lysate corresponding to 500 µg total protein. Immune complexes were harvested using protein A/G-conjugated agarose (Roche), washed five times in lysis buffer and boiled in 2x SDS sample buffer before SDS-PAGE and Western blot. For mass spectrometry, immune complexes precipitated with rabbit anti Baz were

separated by SDS-PAGE, gels were silver stained and selected bands were cut out. Bands were digested with trypsin and analyzed by MALDI-TOF at the ZMMK of the University of Cologne.

Western blotting was done according to standard procedures. Primary antibodies used for Western blotting are listed in the Supplemental Material.

Immunohistochemistry

Embryos were fixed in 4% formaldehyde, phosphate buffer pH 7.4 according to standard procedures. Primary antibodies used for indirect immunofluorescence are listed in the Supplemental Material. Images were taken on a Zeiss LSM 510 Meta confocal microscope and processed using Adobe Photoshop.

Phosphatase inhibition and RNA interference in S2 cells

Inhibition of phosphatases was achieved by incubation of S2 cells with okadaic acid (5 nM) or cantharidin (5 µM or 100 µM) for four h, followed by lysis in lysis buffer supplemented with the same concentration of phosphatase inhibitors.

Knockdown of the different PP2A subunits by RNA interference in S2 cells was done as described (Silverstein et al., 2002; Sathyanarayanan et al., 2004).

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**Figure Legends**

Figure 1. Baz binds to PP2A in vivo. (A) Wild type embryonic extracts were immunoprecipitated with anti Baz antiserum (IP Baz) or the corresponding preimmune serum (IP pre). Blots were probed for Baz, PP2A-A and Mts. (B) PP2A-A antibody was used for immunoprecipitation from extracts of embryos expressing GFP-Baz. Blots were probed for PP2A-A and GFP to detect GFP-Baz. (C, D) Immunoprecipitates of wild type embryonic extracts pulled down with anti aPKC (C) or anti PAR-6 antibody (D). (E – H) The Tws and B56-1 regulatory B subunits of PP2A coimmunoprecipitate with GFP-Baz. S2 cells were cotransfected with GFP-Baz and HA-tagged Tws (E), B56-1 (F), Wdb (G) and PR72 (H). Lysates were precipitated with anti-GFP and probed for

Baz and the HA-tag. Bands of interest are indicated by asterisks. Note that Baz always runs as a series of bands (marked by a bar next to the asterisks) in SDS-PAGE that are generated by proteolytic processing or degradation of the protein. In (E – H) S2 cells transfected only with the HA-tagged B subunits of PP2A were used as negative controls.

Figure 2. The phosphorylation state of three conserved serine residues of Baz can be monitored by phospho-specific antibodies. (A – G) S2 cells expressing GFP-Baz were treated either with DMSO as negative control or with the phosphatase inhibitors okadaic acid (OA) and cantharidin (Canth) at the indicated concentrations. GFP-Baz was immunoprecipitated with anti GFP antibody and the precipitates were subsequently probed with anti Baz (A), anti BazpS151 (B), anti BazpS980 (C) and anti BazpS1085 (D). Lysates were also probed for actin (E), aPKC (F) and aPKCpT422 (G). (H – P) Serine 1085 of Baz is specifically dephosphorylated by a heterotrimeric complex of PP2A-A, Mts and Tws. S2 cells were treated with double stranded RNA corresponding to different subunits of PP2A indicated on top (X RNAi). Double stranded RNA corresponding to GFP was used as negative control. Lysates were subjected to Western blots with the antibodies indicated on the left.

Figure 3. (A – I) Loss of PP2A function leads to complete apical-basal polarity reversal in a fraction of embryonic NBs. Embryos of the indicated genotypes and developmental stages were stained for Baz (red), Mira (blue) and DAPI (turquoise). (J) Quantification of spindle orientation in *wild type* and *PP2A-29B*$^{GE16781}$ mutant embryos. Spindle orientation was determined by drawing a line through the center of the NB that dissected the Baz crescent in the

middle. The angle of that line to a line perpendicular to the plane of the overlying epithelium was measured in increments of 10°. (K – Q) Stage 13 NB polarity is reversed upon overexpression of PAR-1 and Baz and in *leo* mutants. Scale bars = 5 µm. Apical is up.

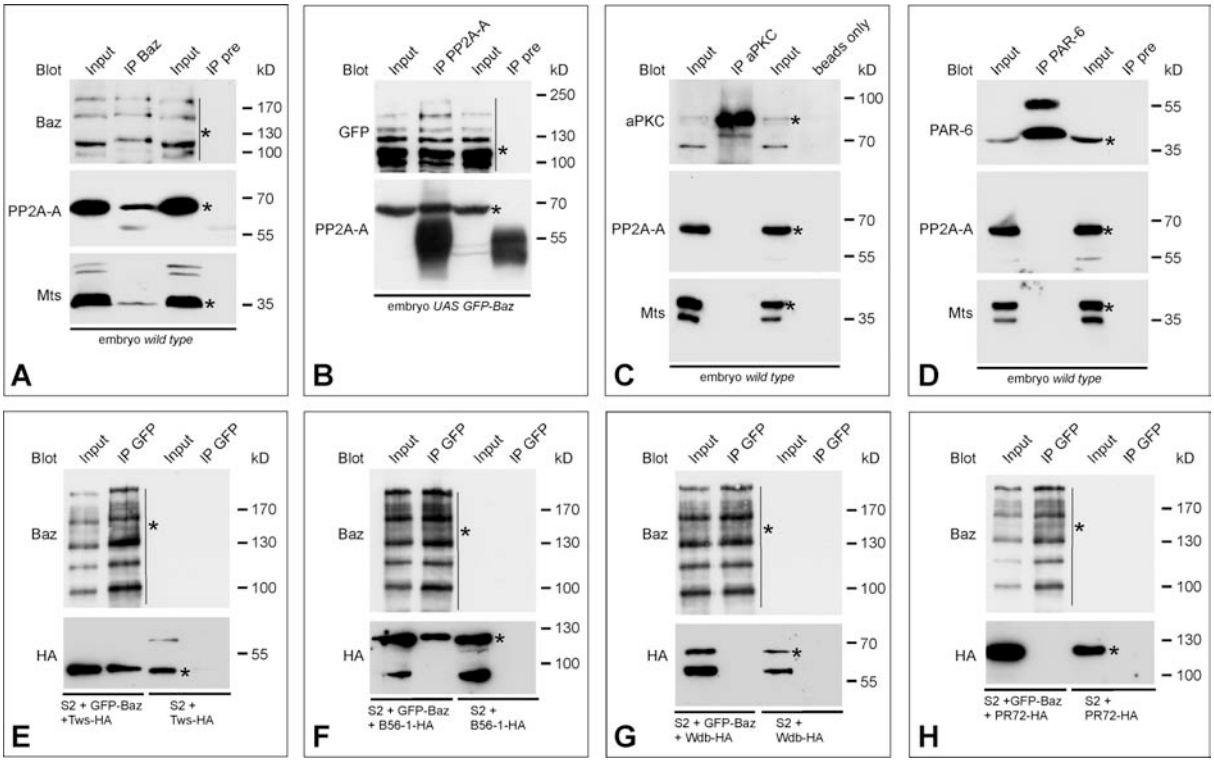
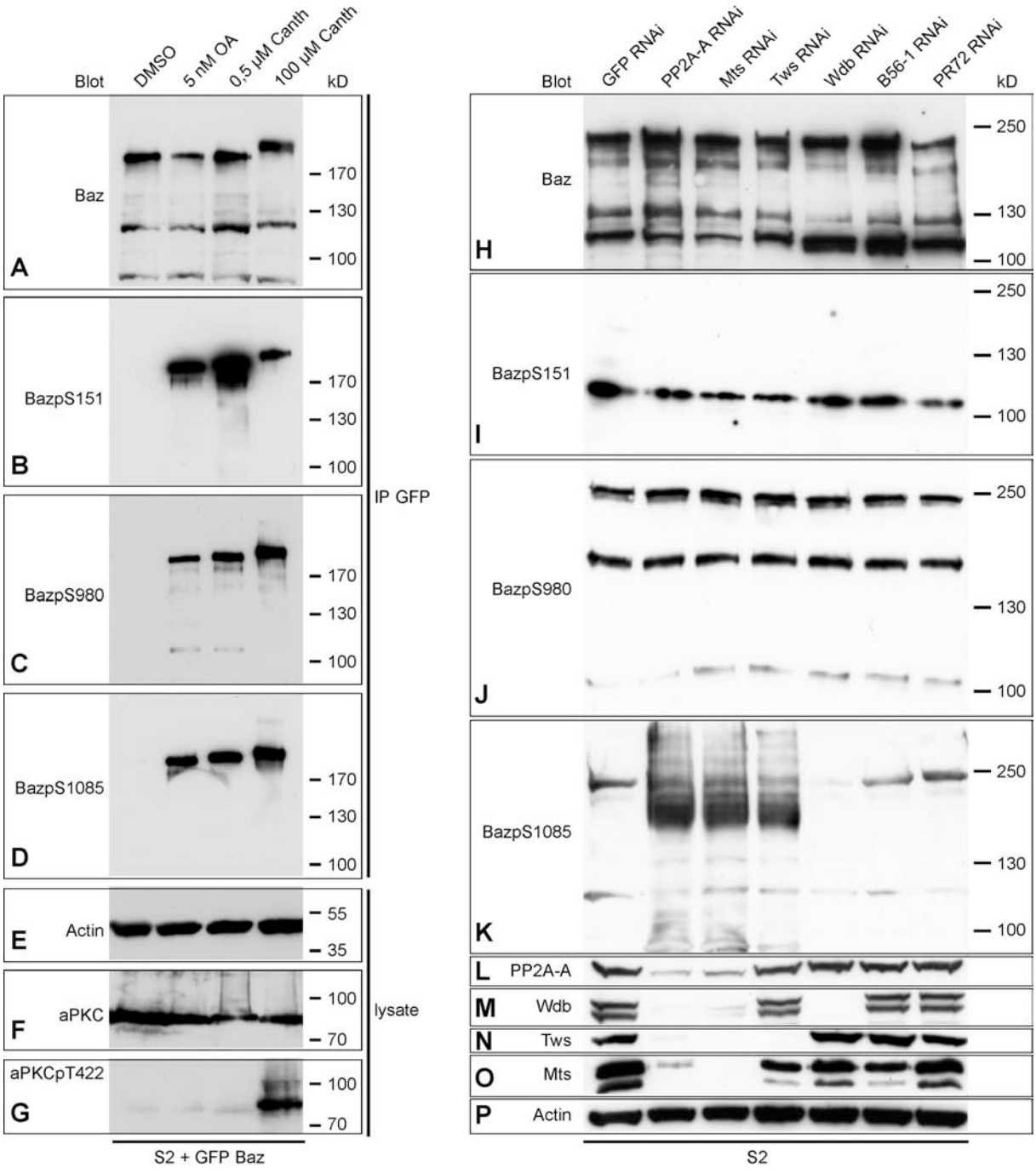

Blot
Input
IP Baz
Input
IP pre
kD
Baz
PP2A-A
Mts
170
130
100
70
55
35
embryo wild type
A
IP PP2A-A
GFP
250
embryo UAS GFP-Baz
B
IP aPKC
beads only
aPKC
C
IP PAR-6
PAR-6
D
IP GFP
HA
S2 + GFP-Baz +Tws-HA
S2 + Tws-HA
E
S2 + GFP-Baz + B56-1-HA
S2 + B56-1-HA
F
S2 + GFP-Baz + Wdb-HA
S2 + Wdb-HA
G
S2 +GFP-Baz + PR72-HA
S2 + PR72-HA
H

Figure 1


Blot
DMSO
5 nM OA
0,5 µM Canth
100 µM Canth
kD
Baz
A
170
130
100
BazpS151
B
BazpS980
C
BazpS1085
D
IP GFP
Actin
E
55
35
aPKC
F
70
lysate
aPKCpT422
G
S2 + GFP Baz
Blot
GFP RNAi
PP2A-A RNAi
Mts RNAi
Tws RNAi
Wdb RNAi
B56-1 RNAi
PR72 RNAi
kD
H
250
I
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K
PP2A-A
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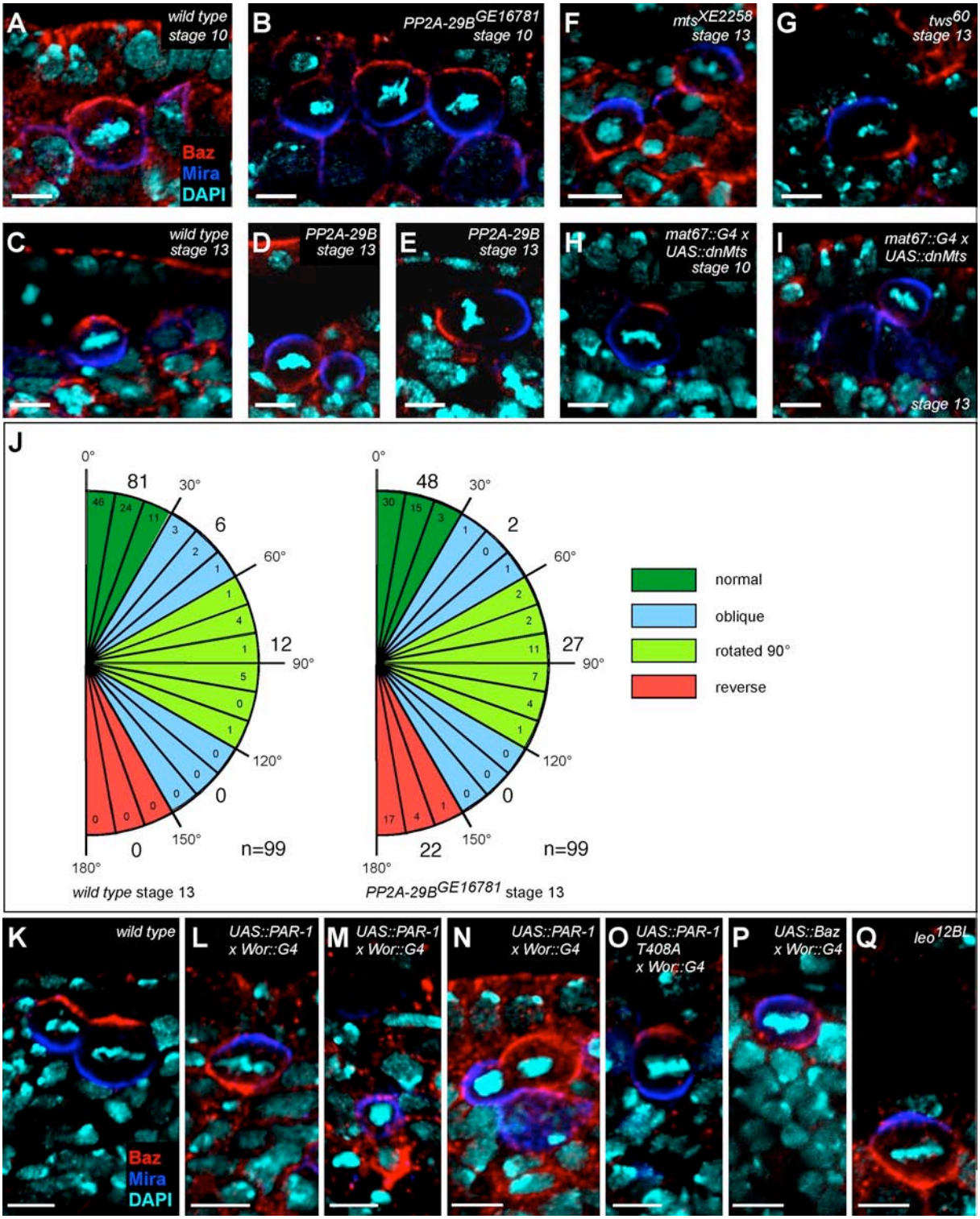
O
P
S2

Figure 2


A
wild type
stage 10
Baz
Mira
DAPI
B
PP2A-29B GE16781
stage 10
F
mts XE2258
stage 13
G
tws 60
stage 13
C
wild type
stage 13
D
PP2A-29B
stage 13
E
PP2A-29B
stage 13
H
mat67::G4 x
UAS::dnMts
stage 10
I
mat67::G4 x
UAS::dnMts
stage 13
J
0°
30°
60°
90°
120°
150°
180°
81
6
12
0
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n=99
wild type stage 13
48
2
27
0
22
n=99
PP2A-29B GE16781 stage 13
normal
oblique
rotated 90°
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wild type
Baz
Mira
DAPI
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UAS::PAR-1
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M
UAS::PAR-1
x Wor::G4
N
UAS::PAR-1
x Wor::G4
O
UAS::PAR-1
T408A
x Wor::G4
P
UAS::Baz
x Wor::G4
Q
leo 12BL

Figure 3

**Table**

| Genotype | NB polarity [%] | | | n |
|---|---|---|---|---|
| **metaphase** | **reversed** | **rotated 90°** | **normal** | |
| *wild type* | 0.6 | 10.6 | 88.8 | 170 |
| *PP2A-29B$^{GE16781}$/PP2A-29B$^{GE16781}$* | 26.5 | 24.9 | 49.6 | 163 |
| UAS::PAR-1 x Wor::GAL4 | 19.0 | 22.1 | 58.9 | 168 |
| UAS::PAR-1T408A x Wor::GAL4 | 2.6 | 12.9 | 84.5 | 162 |
| UAS::Baz x Wor::GAL4 | 23.3 | 20.8 | 55.9 | 168 |
| UAS::BazS1085E x Wor::GAL4 | 20.4 | 25.7 | 53.9 | 161 |
| UAS::BazS1085A x Wor::GAL4 | 4.1 | 14.6 | 81.3 | 167 |
| UAS::BazS151AS1085A x Wor::G4 | 0.6 | 8.1 | 91.3 | 160 |
| *14-3-3leo$^{12BL}$/ 14-3-3leo$^{12BL}$* | 25.8 | 20.5 | 53.7 | 160 |
| *14-3-3leo$^{P1188}$/ 14-3-3leo$^{P1188}$* | 18.4 | 17.9 | 63.7 | 163 |
| *14-3-3ε$^{j2B10}$ / 14-3-3ε$^{j2B10}$* | 6.5 | 11.3 | 82.2 | 165 |
| UAS::Baz, UAS::14-3-3leo x Wor::GAL4 | 7.9 | 12.7 | 79.4 | 161 |
| UAS::Baz, UAS::14-3-3ε x Wor::GAL4 | 5.4 | 13.0 | 71.6 | 168 |
| **telophase** | | | | |
| *wild type* | 0 | 4.9 | 95.1 | 61 |
| UAS::Baz x Wor::GAL4 | 21.1 | 31.6 | 47.4 | 57 |

Table 1. Quantification of NB polarity defects in embryos at stage 13-15. For measurements of NB polarity at metaphase, the angle between a line perpendicular to the plane of the ectodermal epithelium and a line from the center of the NB through the center of the Baz crescent was measured. For telophase NBs, the angle of a line connecting the centers of the two separating daughter cells to the line perpendicular to the plane of the epithelium was measured. Angles between 0° and 30° were scored as normal polarity, angles between 60° and 120° were scored as polarity rotated 90° and angles between 150° and 180° were scored as reversed polarity. Oblique spindle orientations between 30° and 60° or between 120° and 150° were so rare (see Figure 3J) that they were not counted here. The numbers for *wild type* and homozygous mutant *PP2A-29B$^{GE16781}$* NBs shown here differ from

those shown in Figure 3J because they were obtained in independent experiments.

## Supplemental Material

## Supplemental Methods

### Fly stocks

The *PP2A-29B* null allele, the P-element insertion *P{GE16781}* was obtained from GenExel (Daejeon, South Korea). *tws*$^{60}$ was obtained from Tadashi Uemura (Uemura et al., 1993), UAS::dnMts from Amita Sehgal (Hannus et al., 2002; Sathyanarayanan et al., 2004), UAS::PAR-1 and UAS::PAR-1T408A from Bingwei Lu (Sun et al., 2001), UAS::PAR-1-GFP from D. St Johnston (Huynh et al., 2001), UAS::14-3-3ε from J. Botas (Chen et al., 2003), UAS::14-3-3ζ from G. Vorbrueggen (Kockel et al., 1997). UAS::Baz- and UAS::PP2A-29B transgenic fly lines were generated using standard germ line transformation. For overexpression studies with UAS::Baz, five independent P-element insertions were used for each experiment. All other strains used in this study were provided by the Bloomington *Drosophila* stock center.

### Antibodies for Western Blotting

Primary antibodies were used for Western blotting according to standard procedures (Wodarz, 2008) as follows: rabbit anti Baz (1:2000) (Wodarz et al., 1999), rabbit anti PP2A-A (1:2000), rat anti Mts (1:500) (Shiomi et al., 1994), mouse anti GFP (1:1000; Roche 11814460001), rabbit anti-PKCζ C20 (1:2000; Santa Cruz Biotechnology, Inc.), guinea-pig anti PAR-6 (1:1000; Kim et al. submitted), mouse anti HA 12CA5, (1:1000; Roche), rabbit anti BazpS151 (1:100), rabbit anti BazpS980 (1:100), rabbit anti BazpS1085 (1:100), rabbit anti actin A2066, (1:1000; SIGMA), rabbit anti-phospho-PKCζ

T410 (1:1000; Santa Cruz Biotechnology, Inc.), guinea pig anti Wdb (1:2000) (Sathyanarayanan et al., 2004), rat anti Tws (1:500) (Shiomi et al., 1994).

Antibodies for immunohistochemistry

The primary antibodies used were rabbit anti Baz (1:1000) (Wodarz et al., 1999), rat anti Baz (1:500) (Wodarz et al., 1999), guinea-pig anti Mira (1:1000; Kim et al. submitted), rabbit anti PKCζ C20 (1:1000; Santa Cruz Biotechnology, Inc.), rabbit anti Gαi (1:200) (Schaefer et al., 2001), rabbit anti Insc (1:1000) (Kraut and Campos-Ortega, 1996), rabbit anti Pins (1:1000) (Yu et al., 2000), rabbit anti Numb (1:1000) (Rhyu et al., 1994), rabbit anti Pon (1:1000) (Lu et al., 1998), mouse anti Pros MR1A (1:50; DSHB), mouse anti γ-tubulin GTU-88 (1:1000; Sigma), mouse anti GFP 3E6, (1:1000; Invitrogen), rabbit anti BazpS1085 (1:200). DNA was stained with DAPI (Invitrogen). Primary antibodies were detected with secondary antibodies conjugated to Cy2 and Cy3 (Jackson) or Alexa 647 (Invitrogen).

DNA and constructs

The coding regions of PP2A B-subunits (Tws, Wdb, B56-1 and PR72), 14-3-3ε and 14-3-3ζ were amplified by PCR from full-length EST clones (*Drosophila* Genomics Resources Center) as template using the following oligonucleotides (in 5' – 3' orientation):

Tws-for: CACCATGGCCGGTAATGGAGAGG

Tws-rev: GGCTAAAATTTATCCTGAAATATGAAGAGG

Wdb-for: CACCATGTCATCGGGCACGTTTGTG

Wdb-rev: TTAGTTGTCCGCCTTATCCTGTTTG

B56-1-for: CACCATGGTCTTCGGTGCTATGTTG

B56-1-rev: TTAGTAGTTGTTTAGTGTTGTTAATGTGG

PR72-for: CACCATGCAAGCCAATTCGAGCA

PR72-rev: TTAATCGTTTTCCTCGGATATGAGC

14-3-3ε for: CACCATGACTGAGCGCGAGAACAAT

14-3-3ε rev: CGACACGTCCTGATCCTCAAC

14-3-3ζ for: CACCATGTCGACAGTCGATAAGGAAGAG

14-3-3ζ rev: GTTGTCGCCGCCCTCC

The PCR products were cloned into the pENTR vector using the pENTR Directional TOPO Cloning Kit (Invitrogen). Using Gateway Technology (Invitrogen) these constructs were recombined into the pAHW expression vector carrying N-terminal HA under control of the actin-5C promoter (PP2A B-subunits) or into the pAWH vector carrying C-terminal HA under control of the actin-5C promoter (14-3-3 constructs).

Baz full length was cloned into pENTR and was subsequently recombined into different expression vectors (AGW, TGW, PW; Murphy lab, *Drosophila* Genomics Resources Center) using the following primers:

Baz-for: CACCATGAAGGTCACCGTCTGCTTCGGC

Baz-rev: TCACACCTTGGAGGCGTGTG

The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate defined point mutations with full length Baz cDNA in pENTR as template. The following oligonucleotides were used for mutagenesis (mutation underlined):

BazS1085A-for: GAAGTCCTCG<u>GCG</u>TTGGAGTCGCTCCAGACCATGG

BazS1085A-rev: CCATGGTCTGGAGCGACTCCAA<u>CGC</u>CGAGGACTTC

BazS1085E-for: AAGAAGTCCTCG<u>GAG</u>TTGGAGTCGCTCCAGACCATG

BazS1085E-rev: CATGGTCTGGAGCGACTCCAA<u>CTC</u>CGAGGACTTCTT

**Supplemental Figure and Movie Legends**

Figure S1. Identification of PP2A as an interaction partner of Baz. (A) In a yeast two-hybrid screen with the N-terminal 318 aa of Baz as bait we identified an interacting clone containing aa 399 – 480 of PP2A-A. (B) In an independent coimmunoprecipitation experiment, we precipitated Baz from wild type embryonic extract with a polyclonal antibody raised against the N-terminal 297 aa of Baz (Wodarz et al., 1999). Coprecipitating proteins were separated by SDS-PAGE and stained with silver nitrate. A band of 39 kD that was not present in the preimmune control was cut out and analyzed by mass spectrometry. The Mascot search algorithm revealed that the masses of three peptides derived from this band correspond to the masses of three peptides of Mts (marked in red), the 36 kD catalytic subunit of PP2A. While the significance of this hit is not high enough to claim unambiguously that the 39 kD band contains Mts, it is consistent with the size of Mts (36 kD) and with the results of our coimmunoprecipitation analysis using Mts specific antibodies (Figure 1A).

Figure S2. Reversal of apical-basal NB polarity in *PP2A-29B*$^{GE16781}$ mutants. Polarity reversal in *PP2A-29B*$^{GE16781}$ mutants was observed with respect to the apically localized proteins (red) aPKC (A, B), G$\alpha$i (C, D), Insc (E, F) and Pins (G, H) and the basally localized proteins (blue) Numb (I, J), Pon (K, L) and Pros (M, N). DNA was stained with DAPI (turquoise). Scale bars = 5 µm. Apical is to the top in all panels.

Figure S3. (A) PAR-1-GFP localizes predominantly to the basal cortex of mitotic NBs. PAR-1-GFP (green) was expressed in the embryonic nervous

system using the Pros::GAL4 driver line. The stage 13 embryo shown here was also stained for DAPI (turquoise), Baz (red) and Mira (blue). (B) In NBs with reversed polarity that overexpress both PAR-1-GFP and Baz under control of the worniu::GAL4 driver, PAR-1-GFP localizes to the apical cortex opposite of the basally localized Baz. Scale bar = 5 µm. Apical is to the top.

Figure S4. The asymmetry of centrosome size correlates with the localization of Baz in NBs with altered polarity. (A, B) Wild type embryos and (C, D) embryos overexpressing Baz were stained for γ-tubulin (red), Mira (blue), DAPI (turquoise) and Baz (green, only in (C) and (D)). Note that in the wild type NB at metaphase (A) the apical centrosome (arrow) is larger than the basal centrosome (arrowhead). (B) This size asymmetry is more pronounced in ana- and telophase. (C, D) In the NBs with altered polarity due to overexpression of Baz, the centrosome close to the Baz crescent (arrow) is always larger than the centrosome close to the Mira crescent (arrowhead), irrespective of the orientation of the spindle. Images are maximum projections of z-stacks. Scale bar = 5 µm. Apical is up.

Figure S5. Overexpressed wild type Baz gets phosphorylated on S1085. (A) wild type GFP-Baz, (B) GFP-BazS1085A and (C) GFP-BazS1085E were overexpressed in embryos using the engrailed::GAL4 (en::G4) driver line. Stripes of GFP tagged Baz were detectable in all lines in the GFP channel (green) and in stainings using an antibody against Baz that does not discriminate between phosphorylated and unphosphorylated forms (blue). Using the phosphospecifc anti BazpS1085 antibody (red), stripes were only detectable upon overexpression of wild type GFP-Baz (A) and GFP-

BazS1085E (C), but not upon overexpression of GFP-BazS1085A (B). Anterior is to the left and dorsal up. Scale bar = 100 µm.

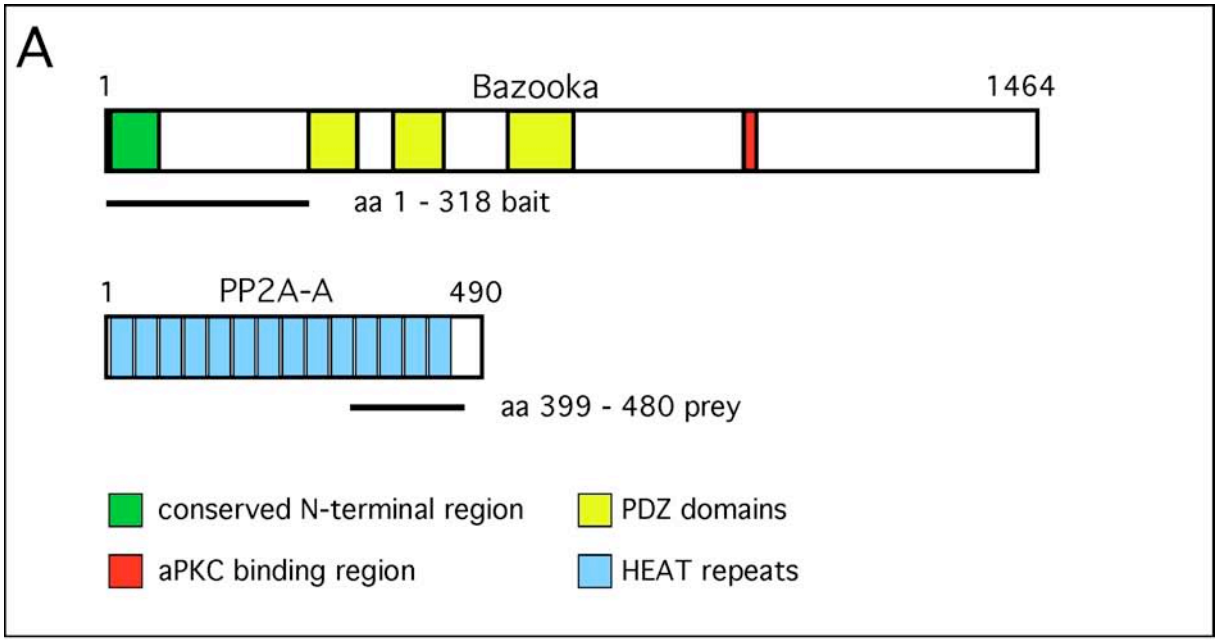
Figure S6. Expression levels of UAS::Baz transgenes are equivalent. The expression levels of the different UAS::Baz transgenes used in this study were compared by Western blot. Equal amounts of embryonic lysate from embryos expressing the respective UAS::Baz constructs under control of daughterless::GAL4 (da::G4) were subjected to Western blot analysis with anti Baz and anti Actin antibodies. Since the Baz proteins encoded by the transgenes were untagged, the signal in the Baz Western is the combination of endogenous Baz and the respective form of Baz encoded by the transgene.

Figure S7. Phosphorylation of S1085 of Baz is inversely correlated with binding of Baz to aPKC and promotes binding of Baz to 14-3-3ε. HA-tagged 14-3-3ε was coexpressed in S2r cells with full length GFP-Baz or with the phosphorylation site mutants GFP-BazS1085A and GFP-BazS1085E. Empty vector instead of the GFP-Baz constructs was used as negative control. Lysates of the transfected cells were immunoprecipitated with an antibody against GFP, followed by Western blot with the indicated antibodies. The input blots of the lysates show that equal amounts of endogenous aPKC and of transfected HA-14-3-3ε were present in all experiments.

Movie S1. Asymmetric NB division in wild type. The movie shows the asymmetric division of a wild type NB expressing PON-GFP. Note that PON-GFP localizes as a basal crescent before segregating into the budding GMC. Apical is up.

Movie S2. Asymmetric NB division with abnormal spindle orientation in a NB overexpressing full length Baz and PON-GFP. Note that PON-GFP localizes as a lateral crescent before segregating into the laterally budding GMC. Apical is up.

Movie S3. Asymmetric NB division with reverse spindle orientation in a NB overexpressing full length Baz and PON-GFP. Note that PON-GFP localizes as an apical crescent before segregating into the apically budding GMC. Apical is up.




B

MATRIX SCIENCE **Mascot Search Results**

**Protein View**

Match to: **PP2A_DROME** Score: **13** Expect: **1.8e+02**
**Serine/threonine-protein phosphatase PP2A OS=Drosophila melanogaster GN=mts PE=1 SV=1**
Found in search of 0287_RCE.txt

Nominal mass ($M_r$): **36016**; Calculated pI value: **5.13**
NCBI BLAST search of PP2A_DROME against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Drosophila melanogaster

Fixed modifications: Carbamidomethyl (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **55**
Number of mass values matched: **3**
Sequence Coverage: **22%**

Matched peptides shown in **Bold Red**

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     1 MEDKATTKDL DQWIEQLNEC NQLTETQVRT LCDKAKEILS KESNVQEVKC
    51 PVTVCGDVHG QFHDLMELFR IGGKSPDTNY LFMGDYVDRG YYSVETVTLL
   101 VALKVRYRER ITILRGNHES RQITQVYGFY DECLRKYGNA NVWKYFTDLF
   151 DYLPLTALVD GQIFCLHGGL SPSIDSLDHI RALDRLQEVP HEGPMCDLLW
   201 SDPDDRGGWG ISPRGAGYTF GQDISETFNN TNGLTLVSRA HQLVMEGYNW
   251 CHDRNVVTIF SAPNYCYRCG NQAALMELDD SLKFSFLQFD PAPRRGEPHV
   301 TRRTPDYFL
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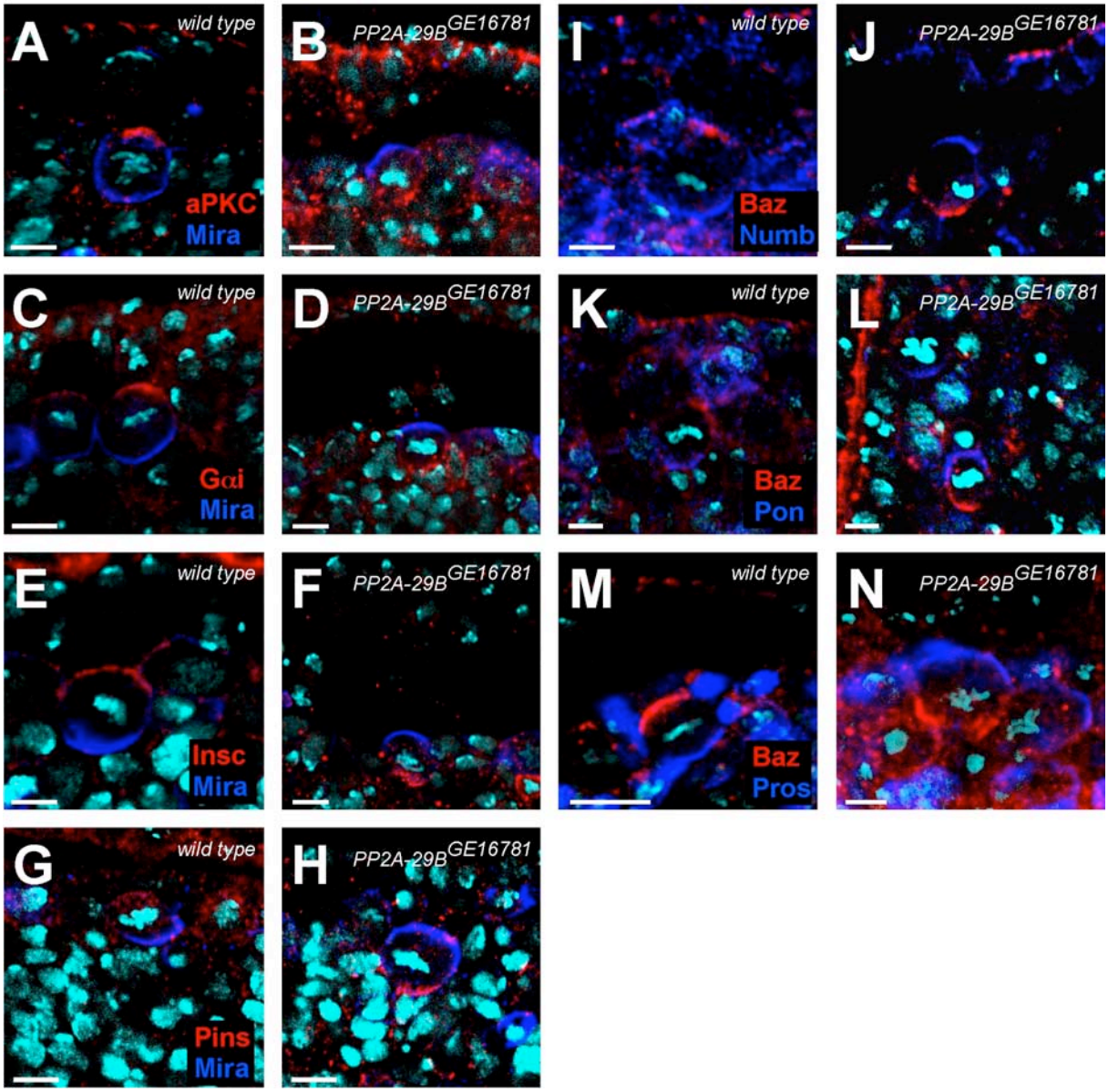
Show predicted peptides also

Sort Peptides By ⊙ Residue Number ○ Increasing Mass ○ Decreasing Mass

| Start - End | Observed | Mr(expt) | Mr(calc) | ppm | Miss | Sequence |
|---|---|---|---|---|---|---|
| 9 - 29 | 2632.2840 | 2631.2768 | 2631.2235 | 20 | 0 | K.DLDQWIEQLNECNQLTETQVR.T |
| 50 - 74 | 2872.3784 | 2871.3711 | 2871.3619 | 3 | 1 | K.CPVTVCGDVHGQFHDLMELFRIGGK.S |
| 215 - 239 | 2662.3510 | 2661.3437 | 2661.2671 | 29 | 0 | R.GAGYTFGQDISETFNNTNGLTLVSR.A |

Figure S1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wild type
aPKC
Mira
B
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I
wild type
Baz
Numb
J
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wild type
Gαi
Mira
D
PP2A-29B GE16781
K
wild type
Baz
Pon
L
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E
wild type
Insc
Mira
F
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M
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H
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Figure S2

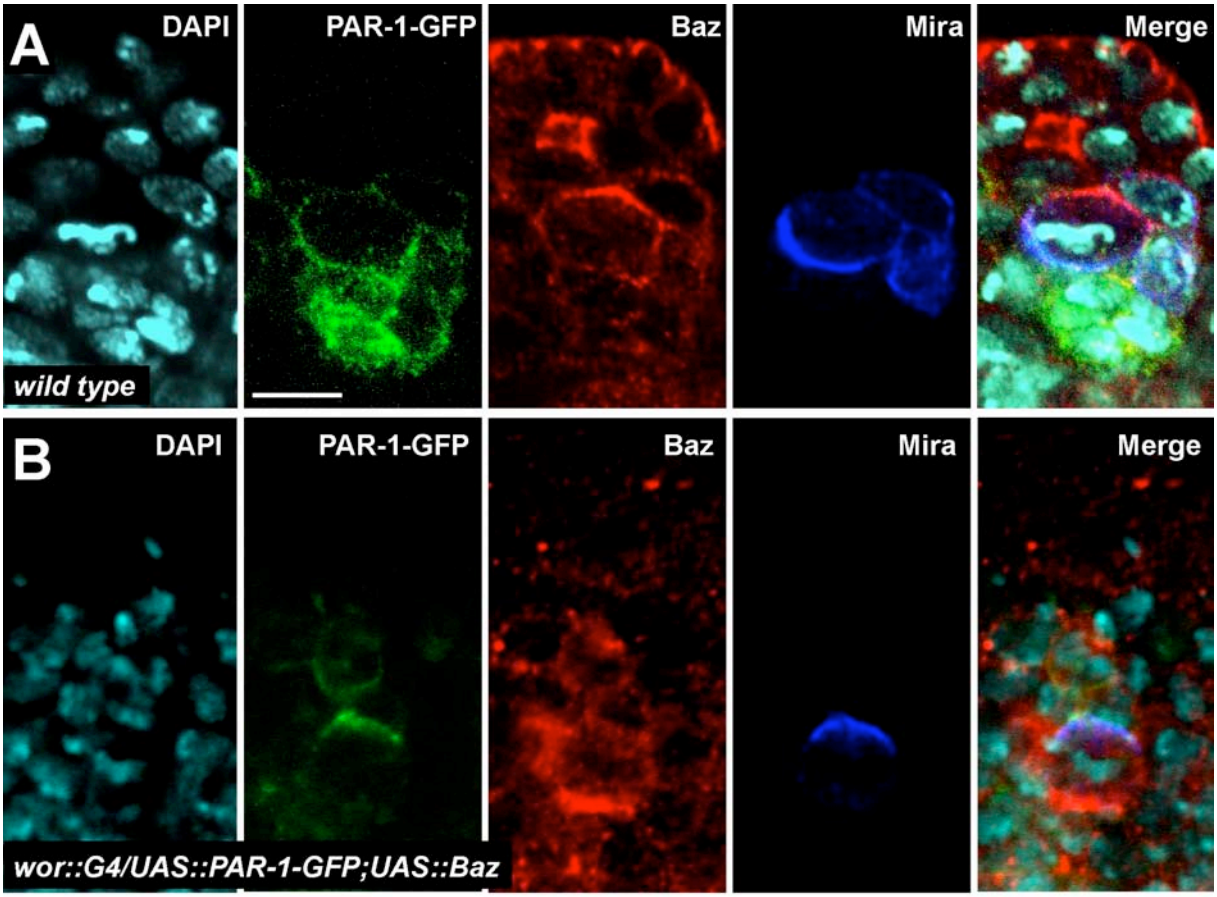
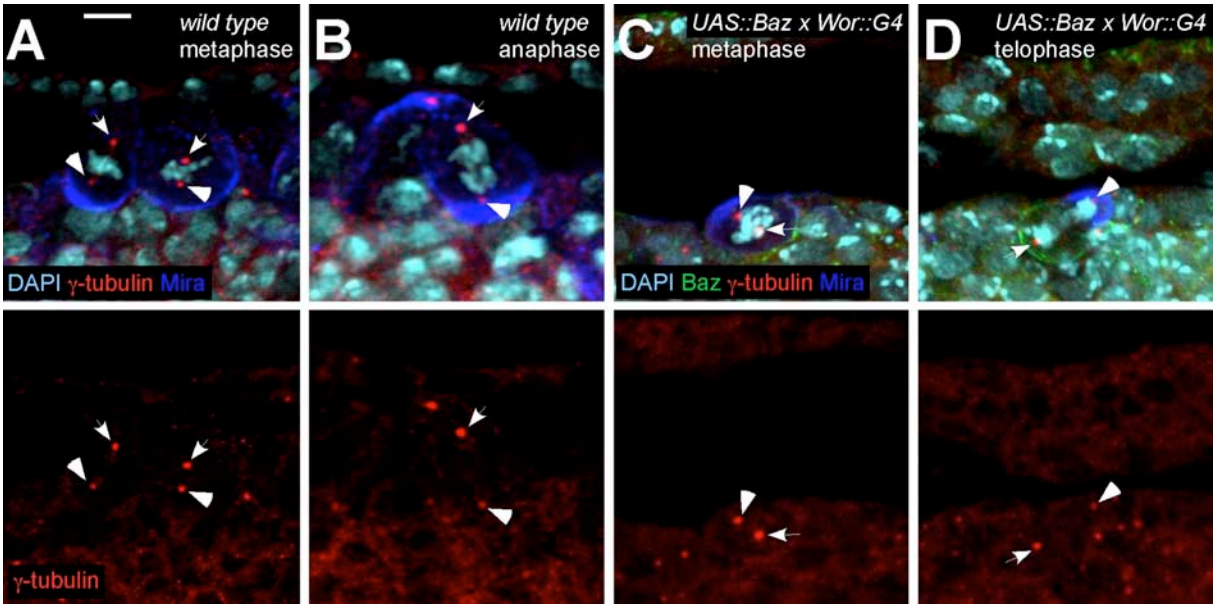

A
DAPI
PAR-1-GFP
Baz
Mira
Merge
wild type
B
DAPI
PAR-1-GFP
Baz
Mira
Merge
wor::G4/UAS::PAR-1-GFP;UAS::Baz

Figure S3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wild type
metaphase
DAPI γ-tubulin Mira
B
wild type
anaphase
C
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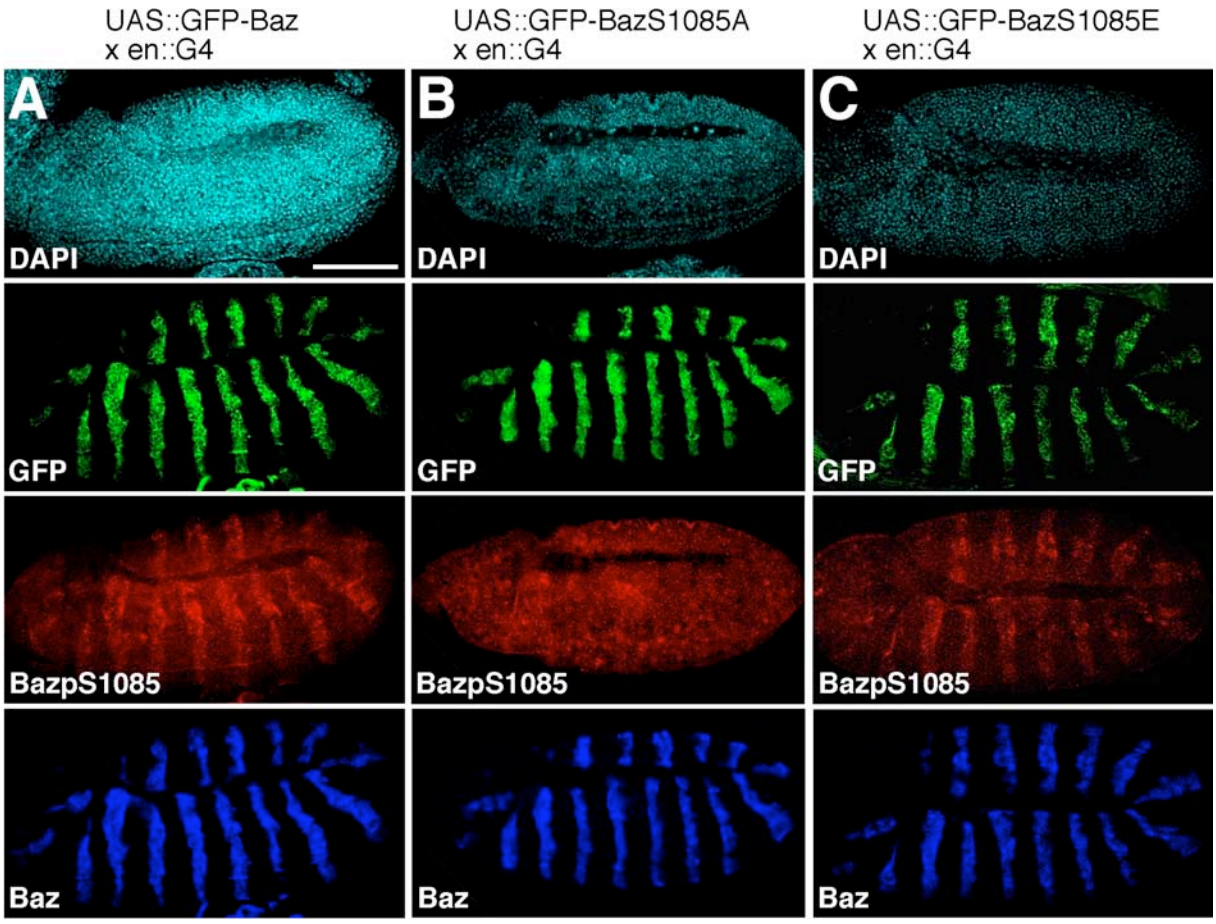
metaphase
DAPI Baz γ-tubulin Mira
D
UAS::Baz x Wor::G4
telophase
γ-tubulin

Figure S4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x en::G4
UAS::GFP-BazS1085A
x en::G4
UAS::GFP-BazS1085E
x en::G4
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B
C
DAPI
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DAPI
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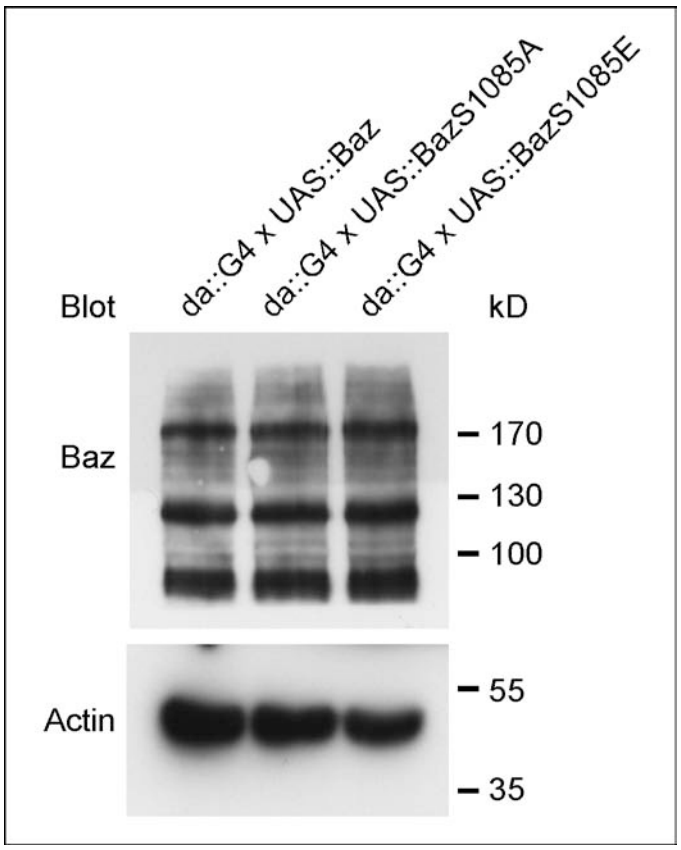
Baz
Baz
Baz

Figure S5


da::G4 x UAS::Baz
da::G4 x UAS::BazS1085A
da::G4 x UAS::BazS1085E
Blot
kD
Baz
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130
100
Actin
55
35

Figure S6

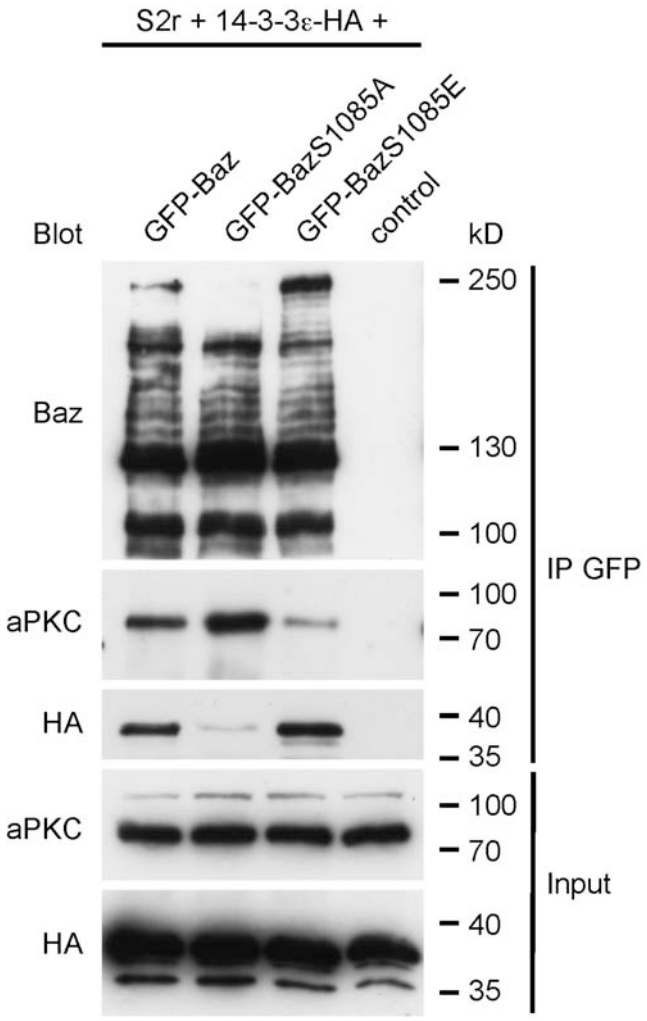
S2r + 14-3-3ε-HA +
GFP-Baz
GFP-BazS1085A
GFP-BazS1085E
control
Blot
kD
Baz
aPKC
HA
aPKC
HA
250
130
100
100
70
40
35
100
70
40
35
IP GFP
Input

Figure S7

## Supplemental References

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## *4.3.* Imapired phosphorylation of Bazooka by aPKC leads *to a dominant negative phenotype*

Here I investigated the interaction between the Bazooka protein and another core component of the PAR/aPKC-complex, aPKC.

I show that in addition to the known aPKC binding domain and the PDZ domains, the poorly conserved linker region between these two domains is essential for binding of aPKC to Baz and phosphorylation of Baz at serine 980..

Impaired phosphorylation of Baz at serine 980 leads to a complete loss of polarity in the embryonic epidermis and in the compound eye, but not in NBs and in the female germ line.

**Michael P. Krahn** and Andreas Wodarz

Author contributions to the work:

**Michael P. Krahn**: All experiments, besides*
writing of the manuscript

**STATUS: MANUSCRIPT IN PREPARATION**

# Impaired phosphorylation of Bazooka by aPKC leads to a dominant negative phenotype

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Running title: Dominant negative version of Bazooka

Keywords: epithelia, aPKC, polarity, PAR proteins, phosphorylation

**Abstract**

The conserved phosphorylation of Bazooka/PAR-3 by aPKC at serine 980 is supposed to be a crucial event in the establishment of cell polarity. However, many details of the interaction between aPKC and Baz as well as the function of their alliance and the phosphorylation event are not yet fully understood.

Here we show that Baz provides several binding sites for aPKC, with the strongest affinity in the PDZ domains and that phosphorylation of S980 increases the binding affinity of Baz to aPKC. A non-phosphorylatable version of Baz exhibits a strong dominant negative phenotype in the embryonic epidermis which can be partially rescued by reduction of aPKC activity and resembles the defects observed in *crb* loss of function alleles.

**Introduction**

Cell polarity is one of the most important features which is acquired throughout the evolution of multicellular organisms. It is not only represented by a variety of cell types and tissues but also controlled by a complicated network of genes. Most of these

Key regulators are highly conserved from worm to man, underlining their importance and success in this complex process.

One of the proteins which functions upstream in the hierarchy controlling cell polarity is Bazooka (Baz), the *Drosophila* homologue of PAR-3, which is the core component of the PAR-3/PAR-6/aPKC complex (Wodarz, 2002; Suzuki and Ohno, 2006). In the *Drosophila* ectodermal epithelium, Baz serves as the first apical cue required for correct targeting of the transmembrane protein Crumbs (Crb) to the apical membrane (Harris and Peifer, 2004; Harris and Peifer, 2005). Loss of Baz does not only result in

mislocalization of Crb and the components of the PAR-complex but leads to a complete loss of cell polarity (Müller and Wieschaus, 1996; Tanentzapf and Tepass, 2003).

Apart from the correct targeting of diverse proteins to their distinct destinations, e.g. the zones of cell-cell contacts or the apical membrane domain, dynamic protein modification has been turned out to be involved in the regulation of cell polarity. Recently it has been shown, that a complex signal cascade triggers the segregation of cell fate determinants into one daughter cell during asymmetric division in neuroblasts (NBs) (Wirtz-Peitz et al., 2008). In mammalian epithelial cells, PAR-1 is phosphorylated by aPKC leading to an inactivation and dissociation of PAR-1 from the apical membrane domain (Hurov et al., 2004). Vice versa, in Drosophila ectodermal epithelial cells, the highly conserved intracellular domain of Crb is phosphorylated by aPKC resulting in a stabilization of Crb protein at the apical membrane domain (Sotillos et al., 2004).

For Baz different phosphorylation sites have been already described to play a crucial role in the establishment and maintenance of polarity: Two conserved serines (S151 and S1085) are phosphorylated by PAR-1, creating a binding site for 14-3-3 proteins (the *Drosophila* homologue of C.elegans PAR-5) and thereby support the exclusion of Baz from the basolateral membrane in epithelial cells and dividing neural stem cells (Benton and St Johnston, 2003)(Krahn et al. 2009).

Baz is also phosphorylated by aPKC (Kim et al. submitted), however the relationship between them seems to be more then a mere kinase-substrate encounter. aPKC binds to the second and third PDZ domain of Baz (Wodarz et al., 2000) and this interaction is supposed to recruit aPKC to the membrane with Baz serving as a scaffold for aPKC. Interestingly, aPKC can also bound to a conserved region at the C-terminal

third of Baz, depicted “aPKC-binding domain”, although all conclusions regarding this interaction are derived from the C.elegans and mammalian homologues PAR-3 and ASIP which are studied excessively in vitro and in vivo (Izumi et al., 1998; Lin et al., 2000; Tabuse et al., 1998).

In mammalian PAR-3 S827 (S980 in Baz) has been shown to be phosphorylated by aPKC resulting in a decreased binding affinity between aPKC and PAR-3 (Nagai-Tamai et al., 2002). Replacement of S827 by alanine, mimicking a not-phosphorylatable version of Baz, results in a loss of cell polarity after calcium switch in mammalian epithelial cells.

However, little to nothing is known if these correlations are also true for Drosophila and if they are significant for all kind of polarized cell types. Therefore in this study we elucidated the association of Baz and aPKC in vitro and in vivo in different polarized cell types and describe the effects of overexpressing versions of Baz which cannot be phosphorylated by aPKC or which mimic a constitutive phosphorylation.

**Results and Discussion**

**Baz binds aPKC by its PDZ domains and its aPKC-binding domain**

In mammalian cells, the minimal region of PAR-3 which is sufficient for binding of aPKC has been reported to be a twenty amino acid stretch at the C-terminal third of the protein (aa 820-840, aa 968-996 in Baz). However, in *Drosophila*, the first association between aPKC and Baz was mapped to the second and third PDZ domain by Yeast-2-Hybrid experiments (Wodarz et al., 2000). In order to clarify which domains are crucial and sufficient for binding of aPKC in vivo, we performed co-

immunoprecipitation with GFP-tagged Baz constructs and endogenous aPKC in S2R+ cells.

Remarkably, deletion of the aPKC-binding domain (BazΔ968-996) leads to a decreased binding of aPKC compared to wt Baz (Figure 1A), but it does not completely abolish this binding. In contrast, deletion of the second PDZ domain (BazΔPDZ2) decreases binding of aPKC and deletion of all three PDZ domains (BazΔPDZ1-3) totally blocks the association between Baz and aPKC. This is in contrast to our previous findings identifying the second and third PDZ domain to be responsible for aPKC binding (Wodarz et al., 2000). However, it fits with data from rescue experiments, indicating that a transgene lacking the third PDZ domain is able to rescue the embryonic lethality of two strong *Baz* null alleles (Krahn et al. submitted). Concomitantly, the isolated C-terminal region of Baz (BazΔ1-905), which includes the aPKC-binding domain, is not capable to pull down aPKC. Surprisingly, deletion of aa 737-945, a rather unconserved region, which has not been assigned any function yet, also blocks aPKC binding, although the mutant protein localizes normally at the apical junctional region in vivo (data not shown).

These finding were supported in vivo by segmental overexpression of UAS::GFP-Baz deletion constructs in wild type embryos using an en::GAL4 driver line, which results in segmental accumulation of aPKC only if Baz but not if BazΔPDZ1-3 are overexpressed (data not shown).

**Phosphorylation of S980 increases binding of aPKC**

For mammalian PAR-3 it has been shown that phosphorylation of S827 (S980 in Baz) decreases the binding affinity of aPKC and Baz (Nagai-Tamai et al., 2002). However,

we find it vice versa: co-immunoprecipitation of full length Baz either as wild type, a not phosphorylatable version (S980A) or a constitutive phosphorylated version (S980E) revealed that binding of aPKC is increased after phosphorylation of S980 and strongly decreased if this serine can not be phosphorylated (Fig. 2A). Concomitantly, addition of a phosphatase inhibitor, preventing hydrolysis of the phosphate groups, also results in a drastic increase of affinity between aPKC and Baz (Fig. 2A). However, this is also true for BazS980E, suggesting that either other phosphorylation sites of Baz apart from S980 are involved or that the phosphorylation state of aPKC itself is increased, leading to a conformational change and enhanced binding affinity to Baz. It has already been shown that for its activation, aPKC must be phosphorylated by PDK-1 (Gao et al., 2001). Therefore, prevention of dephosphorylation would result in an increased activation of aPKC, which in turns could enhance phosporylation of S980 of Baz. Notably, after treatment with a phosphatase inhibitor, a substantial interaction between BazΔPDZ1-3 and aPKC can be detected, whereas it was below the detection level of our assay without inhibitor (Fig.1)

**The unconserved region linking the PDZ domains to the aPKC binding region is crucial for binding of Baz to aPKC and phosphorylation of S980**

Interaction between kinases and substrates are supposed to be rather transient and hard to detect in vivo. Baz has been supposed to function as a scaffold for aPKC, presumably to recruit the kinase activity to the membrane. Therefore, the binding is rather strong. As after deletion of the PDZ domains of Baz no binding to aPKC can be detected in vivo, we investigated if this results in an impaired phosphorylation of S980. Therefore we either wild type GFP-Baz, GFP-BazΔPDZ1-3 or BazΔ737-945

was immunoprecipitated from lysates of transfected cells and blotted with an antibody specific for phosphorylated BazS980. BazS980A was used as a negative control.

Although binding of aPKC can only be demonstrated after addition of phosphatase inhibitor in vivo, BazΔPDZ1-3 is readily phosphorylated at S980 (Fig. 3), indicating, that there exist at least two independent binding sites for aPKC in Baz and that recognition and phosphorylation of the aPKC target motif surrounding S980 is not impaired after deletion of the PDZ domains.

In contrast, deletion of aa 737-945 strongly reduces or even abolishes phosphorylation of S980. BazΔ737-945 is also unable to bind aPKC and fails to rescue the embryonic lethality of *Baz* alleles. Therefore we conclude that this region is not only involved in the binding of aPKC (Fig. 1) but is also crucial for the phosphorylation of S980 and therefore for the functionality of Baz. As this region is only poorly conserved we speculate that the observed negative effect effect might be due to an altered folding of the protein, concealing the binding site for aPKC surrounding S980 and the binding site located in the second PDZ domain.

**Not phosphorylatable Baz results in a dominant negative phenotype**

Nagai-Tamai et al. already showed for mammalian epithelial cells that overexpression of a phosphorylation-defective version of Baz (S827A) results in an impaired formation of cell polarity after calcium switch (Nagai-Tamai et al., 2002). To test if this is also true for *Drosophila* and in particular if the polarity of all polarized cell types is affected, we expressed UAS::GFP-BazS980A with tissue specific driver lines. In epithelial cells of the epidermis, the effect of BazS980A overexpression is drastic: embryos die in late embryogenesis with a totally messed up morphology and

no cuticle secreted (Fig. 4A, Fig. 5, supplemental movie 2). Interestingly, epidermal structures are nearly totally destructed whereas the intestine is proliferating and expanding, forming large bulbs (Fig. 4A, arrow, Fig. 4A' asterisk). In these structures, BazS980A is mostly correctly localized to the apical (luminal) junctional region (Fig. 4A', arrow) with some aggregates mislocalized to the basal site (Fig. 3A', arrowhead). Similarly, other polarity markers as DE-cad, Dlg (Fig. 4A'), aPKC, Arm and Dystrophin (data not shown) are correctly localized in this tissue. Notably, instead of being restricted to the subapical region, BazS980A-GFP and DE-Cad are frequently found to be spread over the entire free apical membrane. Crb can not be detected in these structures (Fig. 4A and data not shown), supporting the hypothesis that the bulb-like structures are originated from the midgut.

Analyzed in more detail, it is obvious that in the epidermis of earlier stages, BazS980A localizes predominately to the apical junctional region (Fig 4A''), whereas in later stages, it forms cytoplasmic and cortical aggregates, which also include Crb, PAR-6 (Fig. 4A'''), aPKC and DE-Cad (data not shown). Striking is the disturbance of Crb in earlier stages when BazS980A is expressed: Although Baz S980A is still cortical and without bigger aggregates, Crb is to some extent depleted from the membrane (Fig. 4''). Taken together these data indicate that the overexpression of BazS980A severely disturbs the polarity in the epidermal epithelium.

Surprisingly, in the follicle epithelium and in the oocyte, the polarity is not affected upon BazS980A overexpression and the transgene localizes correctly to the ZA and to the anterior cortex, respectively (Fig. 4B, C). Concomittantly, female flies overexpressing BazS980A in the germ line lay normal eggs.

Similarly, in embryonic NBs, BazS980A localizes to the apical membrane as its wild type counterpart, without affecting the localization of cell fate determinants, spindle orientation or the asymmetric cell division (Fig. 4D and data not shown).

Finally, overexpression of BazS980A in the eye using GMR::G4 also results in a drastic phenotype: Upon overexpression of wild type Baz in the eye, a "rough eye" phenotype can be observed, indicating that the polarity of the omatidia is disturbed. This phenomenon is dramatically increased upon overexpression of BazS980A, leading to a nearly complete destruction of the compound eye (Fig. 4E).

**Membrane association is crucial for the dominant negative phentoype of BazS980A overexpression**

In order investigate which of the domain of Baz are involved in the dominant negative phenotype of BazS980A overexpression, we tested different deletion constructs of Baz with the S980A mutation: First, deletion of the conserved N-terminal oligomerization domain (BazΔ1-317S980A) does not diminish the dominant negative phenotype of S980A. The same is true if all three PDZ domains are deleted together with the S980A mutation. Interestingly, in contrast to BazΔPDZ1-3, BazΔPDZ1-3S980A is capable to bind aPKC in S2 cells (Fig. 1) and segmental overexpression of BazΔPDZ1-3S980A results in a strong accumulation of aPKC (Fig. 5).

aPKC has been reported to act as a constitutive active version, if it is tethered to the membrane by addition of a prenylation signal ($aPKC^{CAAX}$)(Sotillos et al., 2004). We have shown recently, that the C-terminal region of Baz is crucial for the membrane localization of the protein (Krahn et al. submitted). To test, whether membrane association of Baz is essential for the S980A phenotype, we overexpressed a C-

terminal truncated version of Baz carrying the S980A mutation (BazΔ1097-1464S980A), which shows a strongly decreased membrane association (Krahn et al. submitted and data not shown). Indeed, overexpression of BazΔ1097-1464S980A does not affect cell polarity or viability of the embryos.

However, testing the isolated C-terminus including the aPKC-binding region with the S980A mutation (BazΔ1-904S980A), we were not able to detect any defects in the embryonic epidermis. This might be due to the fact, that BazΔ1-904 does not entirely localizes to the ZA as full length Baz but it also accumulates to some extent in the cytoplasma and at the basolateral membrane (Krahn et al. submitted).

Taken together, our data indicate that the effects of BazS980A overexpression are facilitated by the membrane-association of Baz but are not dependent on the N-terminal oligomerization domain or the PDZ domains.

Therefore one explanation of the mechanism underlying the dominant negative phenotype of BazS980A overexpression could be that physiologically, aPKC is recruited to the membrane by Baz, phosphorylates S980 resulting in an increased binding to this domain, which in turn results in a block of aPKC kinase activity. Thereby Baz would serve not only as a scaffold for aPKC but also as a trigger for aPKC activity. If S980 is not phosphorylatable anymore, the inhibitory effect of Baz would be omitted, releasing untriggered aPKC activity.

**Cuticle defects of BazS980A overexpression resemble *crb* mutant phenotypes**

An intact cell polarity is the prerequisite for the secretion of a physiological cuticle by the embryonic epidermis at the end of embryogenesis. Alleles of several genes involved in the regulation of cell polarity show defects in cuticle secretion (Knust et

al., 1993; Kuchinke et al., 1998; Petronczki and Knoblich, 2001; Tepass et al., 1996; Wieschaus et al., 1984; Wodarz et al., 2000). Therefore we investigated the effect of BazS980A overexpression on the cuticle secretion. As shown in Fig. 6, only minimal debris of cuticle can be detected, a defect which is strikingly similar to the phenotype of *crb* loss of function alleles (Knust et al., 1993), which is very characteristically, leading to the denotation of the gene "*crumbs*" (Knust et al., 1993).

This raises the question if the overexpression of BazS980A somehow affects either the stabilization/degradation of the Crb protein or its functionality. As no differences in protein levels of Crb upon BazS980A overexpression compared to wild type lysate or wild type Baz overexpression can be detected (data not shown), we focused on the second hypothesis.

**The dominant negative phenotype of BazS980A-overexpression is partly rescued by reduction of aPKC activity**

Crb localization and functions is dependent on the correct formation of the PAR/aPKC complex (Bilder et al., 2003), which has recently been described in more detail: aPKC phosphorylates two threonines in the conserved intracellular domain of Crb, which is crucial for proper localization and function of the protein (Sotillos et al., 2004). Replacement of the involved residues by alanine leads to a loss of membrane association of Crb. Concomitantly, overexpression of a dominant negative, kinase dead version of aPKC ($aPKC^{CAAX\ DN}$) also leads to a downregulation of Crb, whereas overexpression of an active, membrane bound aPKC form ($aPKC^{CAAX}$) results in an expansion of the Crb-positive apical domain to the basolateral membrane (Sotillos et al., 2004).

In order to elucidate the interaction between BazS980A overexpression and aPKC activity, we tested whether reduction of aPKC activity can rescue the phenotype of BazS980A overexpression. Indeed, loss of one copy of aPKC decreases the severeness of the BazS980A overexpression phenotype in cuticle preparations (Fig. 6), although the dominant negative phenotype is not fully rescued and the embryonic lethality is still 100%.

Taken together we have shown that Baz serves as a multiple scaffold for aPKC by binding to aPKC via its second PDZ domain and the aPKC binding domain. Although not conserved, the region between these two identified binding region is crucial for aPKC association and phosphorylation of S980. This phosphorylation serves as a trigger to increase the binding to aPKC, presumably to inhibit its cortical kinase acitivity. The key question is, what is the substrate for aPKC? aPKC has already been reported to phosphorylate several regulators of polarity: PAR-1, which is thereby inactivated and released from the membrane (Hurov et al., 2004), lgl, which is also inactivated (Betschinger et al., 2005; Betschinger et al., 2003; Ponting, 1997; Yamanaka et al., 2003), numb, leading to a restriction to the basal cortex of dividing NBs (Smith et al., 2007; Wirtz-Peitz et al., 2008) and Crb (Sotillos et al., 2004).

Taking in account first the fact that the most predominant phenotype of BazS980A overexpression is found in the eye and in the embryonic epidermis; second, the polarity of the oocyte and embryonic NBs are not affected, third, Crb-negative luminal structures, presumably the midgut, proliferate in otherwise totally messed up embryos leads to the conclusion that Crb is the most likely candidate of the known substrates of aPKC. In fact, the cuticle phenotype of BazS980A overexpression is identical to that of *crb* mutant. However, it still remains unclear, how Crb should be degraded upon BazS980A overexpression: Given the fact that Baz recruits aPKC to

the membrane and regulates its kinase activity via its aPKC binding domain, the S980A mutation should lead to an increase in aPKC kinase activity, which in turn excessively phosphorylates Crb. Following this hypothesis, the resulting phenotype would be instead of a loss of function of *crb* a gain of function, reflected by ectopic cuticle and a spatially multilayered ectodermal epithelium (Wodarz et al., 1995).

Therefore, BazS980A-overexpression must somehow provide an increased feedback loop to restrict aPKC activity. One possibility would be that the S980A mutation results in a conformational change of the Baz protein, which now binds differentially to aPKC, not providing a scaffold for membrane tethering but inhibiting its activity. Another possibility would be that by this conformational change, Baz exposes a different binding site for an up to now unidentified protein, which in turn inhibits aPKC. One candidate for inhibition of aPKC is PAR-6, although we have not yet any evidence that S980A increases the binding of PAR-6.

**Materials and Methods**

Fly stocks and genetics

Transgenic flies carrying UAS::GFP-Baz constructs were generated using standard germ line transformation. The following GAL4 driver lines were used for expression of the transgenes in different tissues: daughterless::GAL4 (da::G4) (Wodarz et al., 1995), worniu::GAL4 (wor::G4), nanos::GAL4 (nos::G4). Cuticle preparations werde performed according to standard protocols.

DNA and constructs

Cloning of wild type Baz in pEntr and destination vectors has been described previously (Krahn et al. 2009). For BazS980A, following oligonuceotides were used (in 5'-3' orientation, mutation underlined) :

BazS980A-for: GATGCATTGGGACGACGCGCCATCTCTGAGAAG
BazS980A-rev: CTTCTCAGAGATGGCGCGTCGTCCCAATGCATC
Cloning of all other constructs have been described elsewhere (Krahn et al. submitted).

Immunoprecipitation and Western Blot

For immunoprecipitations, embryos from an overnight collection were dechorionated and lysed in lysis buffer (1% Triton X-100, 150mM NaCl, 1mM CaCl2, 1mM MgCl2, 25mM Hepes pH 7.44) supplemented with protease inhibitors. After centrifugation, 2 µl of mouse anti GFP (Invitrogen) were added to cell lysate corresponding to 500 µg total protein. Immune complexes were harvested using protein G-conjugated agarose (Roche), washed five times in lysis buffer and boiled in 2x SDS sample buffer before SDS-PAGE and Western blot.

Primary antibodies were used for Western blotting according to standard procedures (Wodarz, 2008) as follows: mouse anti GFP (1:1000; Roche 11814460001), rabbit anti-PKCζ C20 (1:2000; Santa Cruz Biotechnology, Inc.).

Immunohistochemistry

Embryos and ovaries were fixed in 4% formaldehyde, phosphate buffer pH 7.4. The primary antibodies used were guinea-pig anti Mira (1:1000; Kim et al. submitted), rabbit anti PKCζ C20 (1:1000; Santa Cruz Biotechnology, Inc.), rat anti DE-Cadherin DCAD2 (1:50; Developmental Studies Hybridoma Bank, DSHB), mouse anti Dlg 4F3 (1:50; DSHB), rabbit anti Staufen (1:1000) (St Johnston et al., 1991), mouse anti

Gurken 1D12 (1:10, DSHB), mouse anti Crb (1:10, DSHB), mouse anti GFP 3E6 (1:1000; Invitrogen). DNA was stained with DAPI (Invitrogen). Secondary antibodies conjugated to Cy2 and Cy3 were obtained from Jackson Laboratories. Secondary antibodies conjugated to Alexa 647 were obtained from Invitrogen. Images were taken on a Zeiss LSM 510 Meta confocal microscope and processed using Adobe Photoshop.

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**Figure Legends**

Figure 1. The PDZ domains of Baz are crucial for binding of aPKC. Endogenous aPKC protein was co-immunoprecipitated with GFP-Baz variants from transfected S2R cells.

Figure 2. Phosphorylation of S980A increases binding of aPKC to Baz. S2R cells transfected with GFP-Baz constructs werde treated with DMSO as negative

control or 10µM cantharidin for 2h. GFP-Baz proteins were imunoprecipitated with an anti-GFP antibody and blotted against aPKC and GFP.

Figure 3. Phosphorylation of S980A is impaired after deletion of aa 737-945. Different GFP-Baz constructs were expressed in S2R cells and immunoprecipitated. Proteins were dissolved on SDS-PAGE and blotted against GFP and pS980.

Figure 4. Overexpression of BazS980A results in severe polarity defects of the embryonic epidermis. GFP-BazS980A was overexpressed in different polarized cell types using distinct driver lines. (A) Upon ubiquitous overexpression using da::G4, the morphology of embryos in later stages is severely disturbed. (A') Cavernous structure, presumably representing midgut tissue. (A''-A''') Segmental overexpression of GFP-BazS980A with en::G4. In early stages, BazS980A still localizes at the cortex, whereas it forms aggregates in later stages. Crb expression is decreased in early stages and in later stages included in BazS980A positive aggregates. (B) Expression of BazS980A in the oocyte shows wild type localization at the anterior cortex, whereas Staufen (Stau) localizes posterior. Gurken (Grk) marks the dorso-posterior pol. (C) BazS980A localizes normally in embryonic NBs at the apical cortex, overlapping with aPKC. Miranda (Mira) accumulates at the basal pole. (D) In the eye, overexpression of BazS980A results in a completely dirsturbed morphology, compared to a discrete rough-eye phenotype of wild-type Baz overexpression.

Figure 5. BazΔPDZ1-3S980A but not BazΔPDZ1-3 is able to recruit aPKC in vivo. In order to assay in vivo recruitment of aPKC, aPKC was costained. BazΔPDZ1-3 and BazΔPDZ1-3S980A were segmental overexpressed using en::G4.

Figure 6. Embryos overexpressing BazS980A fail to secrete a physiological cuticle Cuticle preparation of embryos overexpressing BazS980A, wild type Baz or BazS980A with only one copy of aPKC.

Figure 1

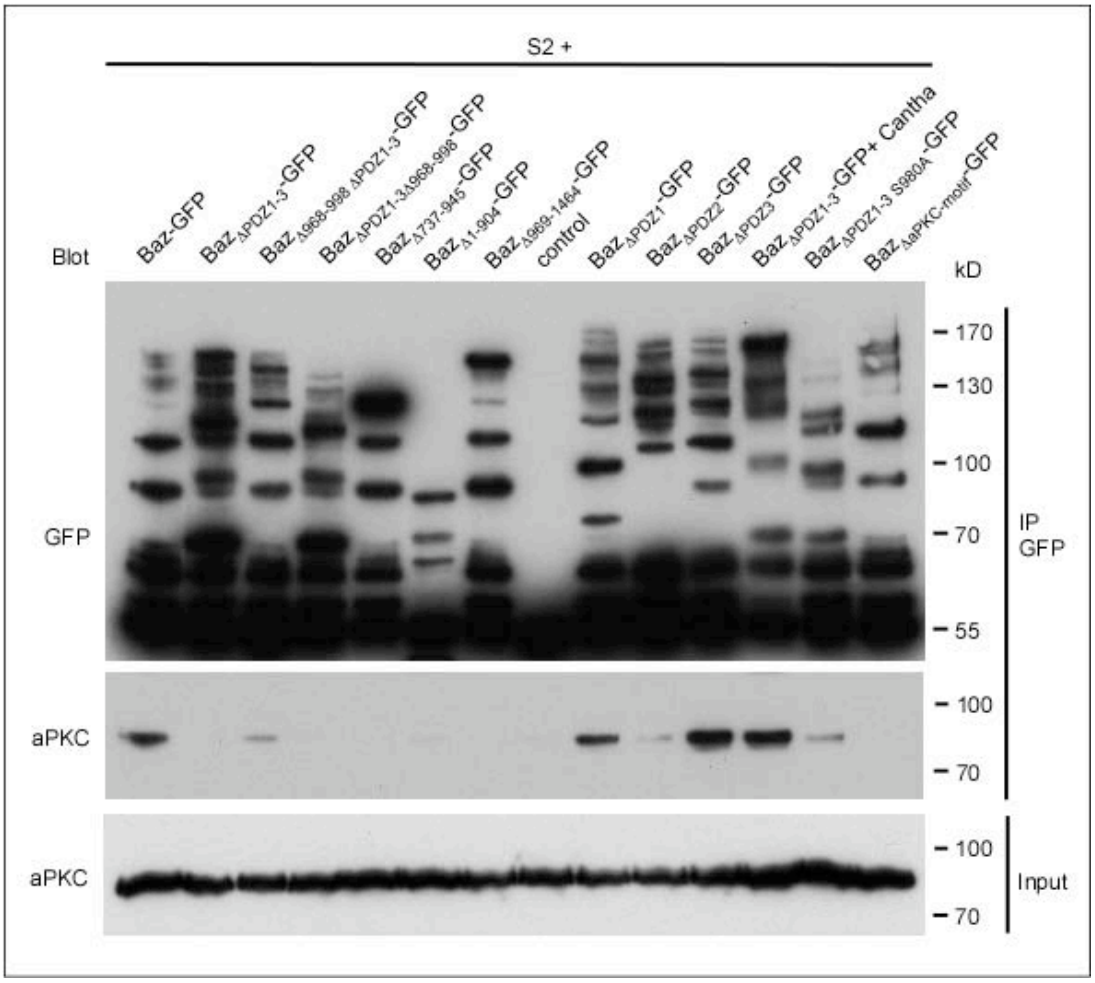

S2 +
Baz-GFP
Baz ΔPDZ1-3-GFP
Baz Δ968-998 ΔPDZ1-3-GFP
Baz ΔPDZ1-3Δ968-998-GFP
Baz Δ737-945-GFP
Baz Δ1-904-GFP
Baz Δ969-1464-GFP
control
Baz ΔPDZ1-GFP
Baz ΔPDZ2-GFP
Baz ΔPDZ3-GFP
Baz ΔPDZ1-3-GFP+ Cantha
Baz ΔPDZ1-3 S980A-GFP
Baz ΔaPKC-motif-GFP
Blot
kD
GFP
170
130
100
70
55
IP GFP
aPKC
100
70
aPKC
100
70
Input

Figure 2

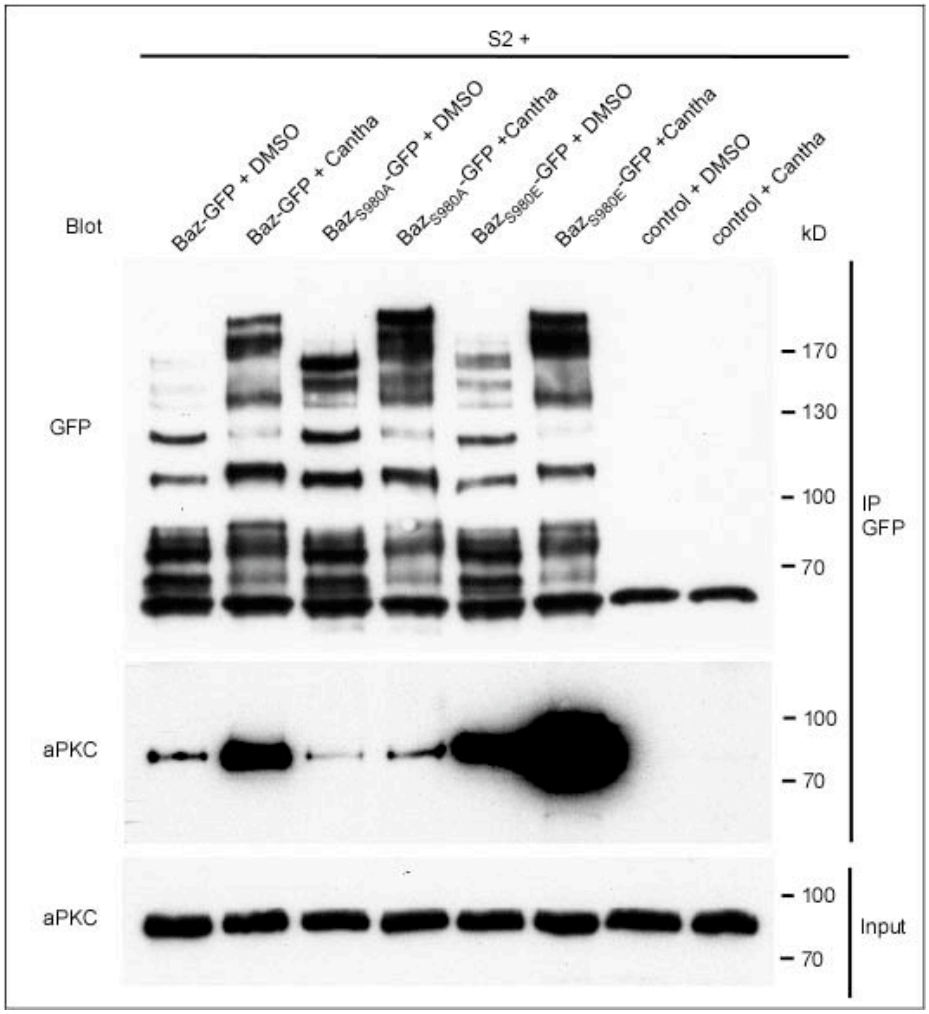

S2 +
Baz-GFP + DMSO
Baz-GFP + Cantha
Baz$_{S980A}$-GFP + DMSO
Baz$_{S980A}$-GFP +Cantha
Baz$_{S980E}$-GFP + DMSO
Baz$_{S980E}$-GFP +Cantha
control + DMSO
control + Cantha
Blot
kD
GFP
aPKC
aPKC
170
130
100
70
100
70
100
70
IP GFP
Input

Figure 3

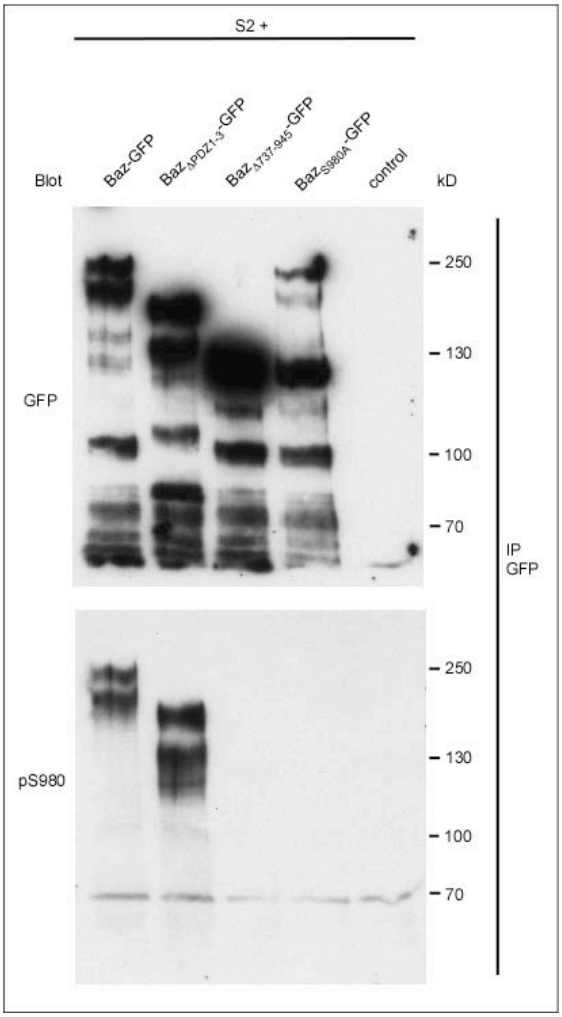
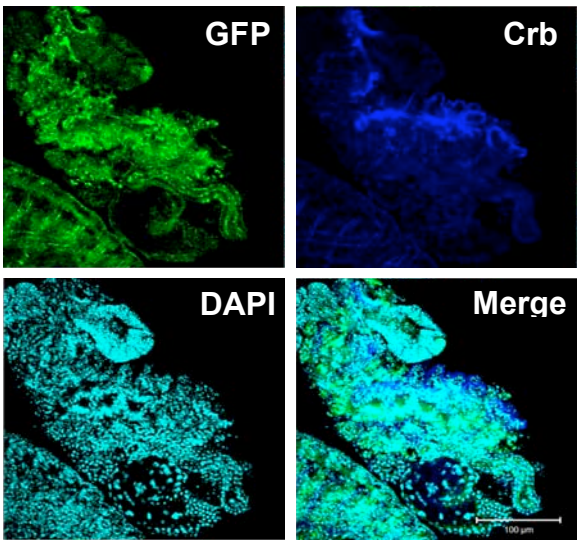
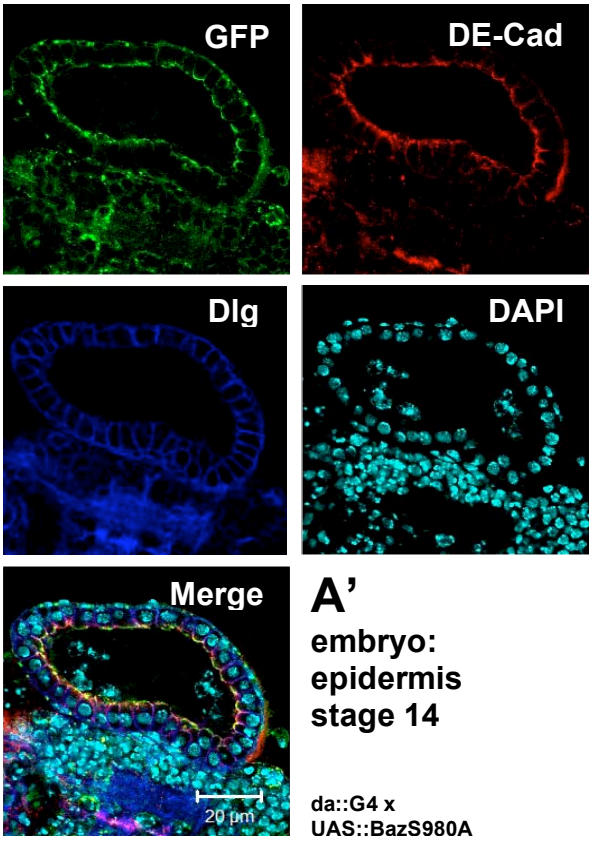

S2 +
Blot
Baz-GFP
Baz$_{\Delta PDZ1-3}$-GFP
Baz$_{\Delta 737-945}$-GFP
Baz$_{S980A}$-GFP
control
kD
GFP
250
130
100
70
IP
GFP
pS980
250
130
100
70

Figure 4

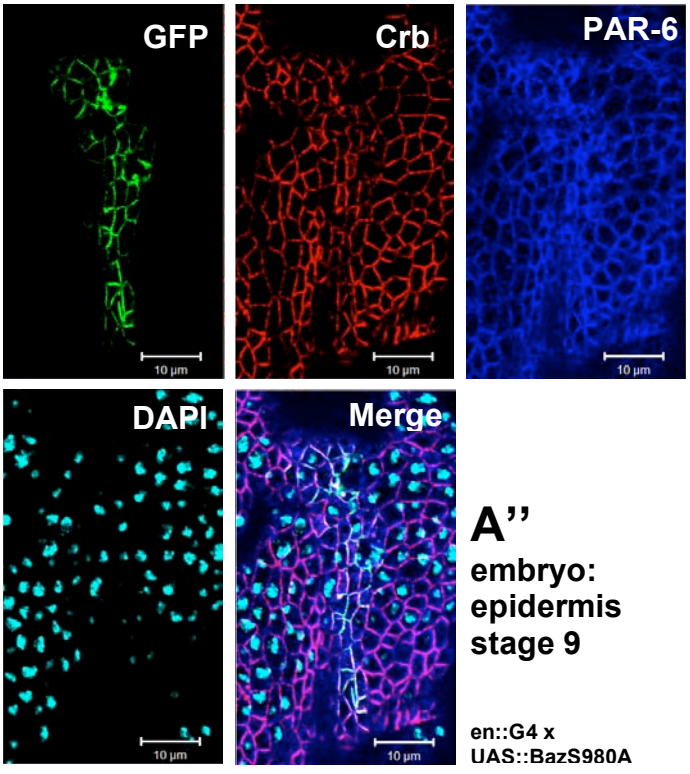



**A** embryo: overview stage 14
da::G4 x UAS::BazS980A



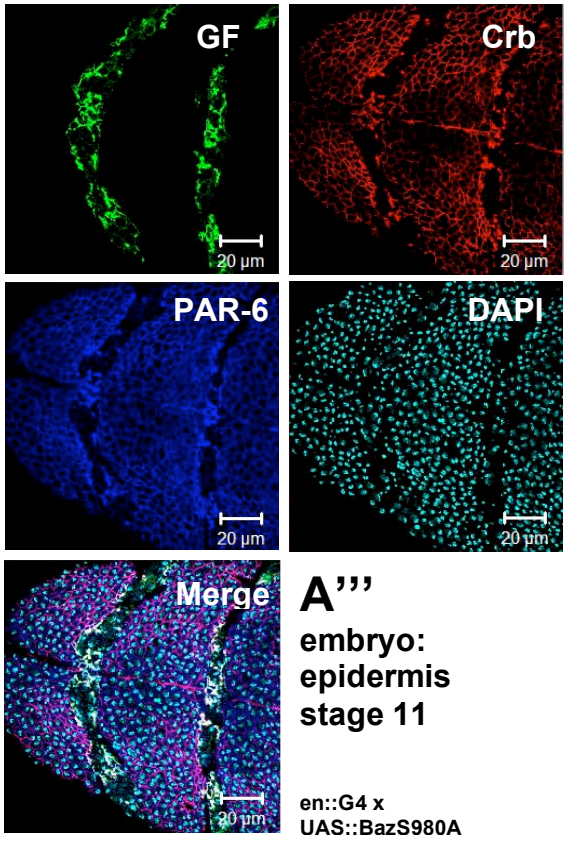

**A'**
embryo: epidermis stage 14

da::G4 x UAS::BazS980A



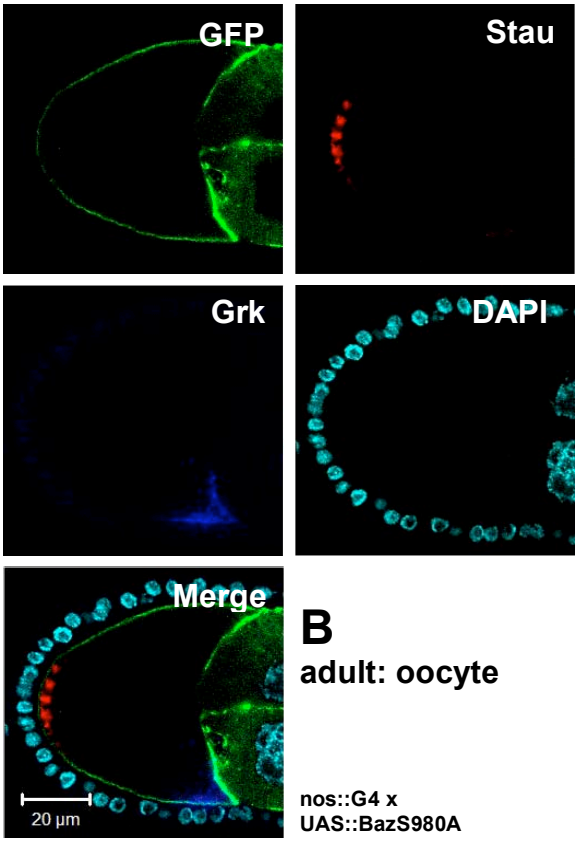

**A''**
embryo: epidermis stage 9

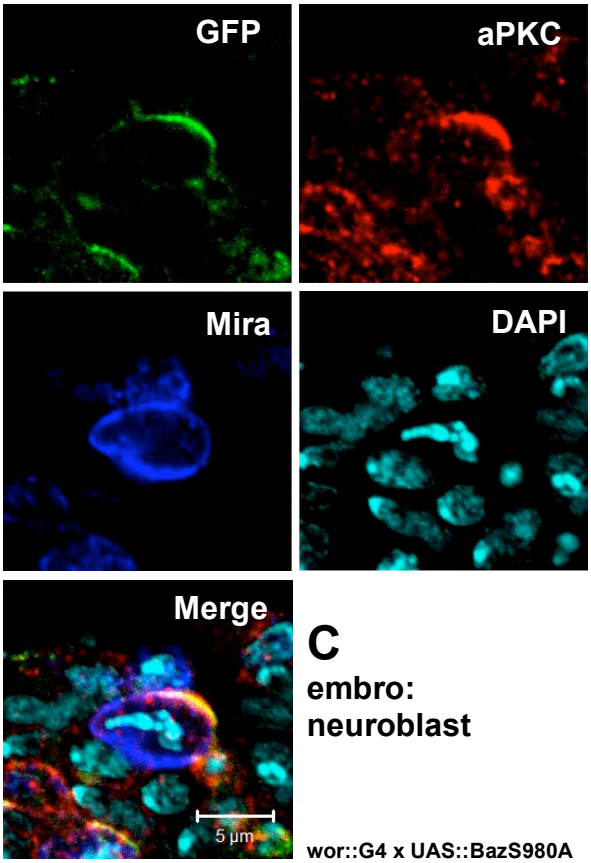
en::G4 x UAS::BazS980A




**A'''**
embryo: epidermis stage 11

en::G4 x UAS::BazS980A


GFP
Stau
Grk
DAPI
Merge
20 µm
B
adult: oocyte
nos::G4 x
UAS::BazS980A


GFP
aPKC
Mira
DAPI
Merge
5 µm
C
embro:
neuroblast
wor::G4 x UAS::BazS980A

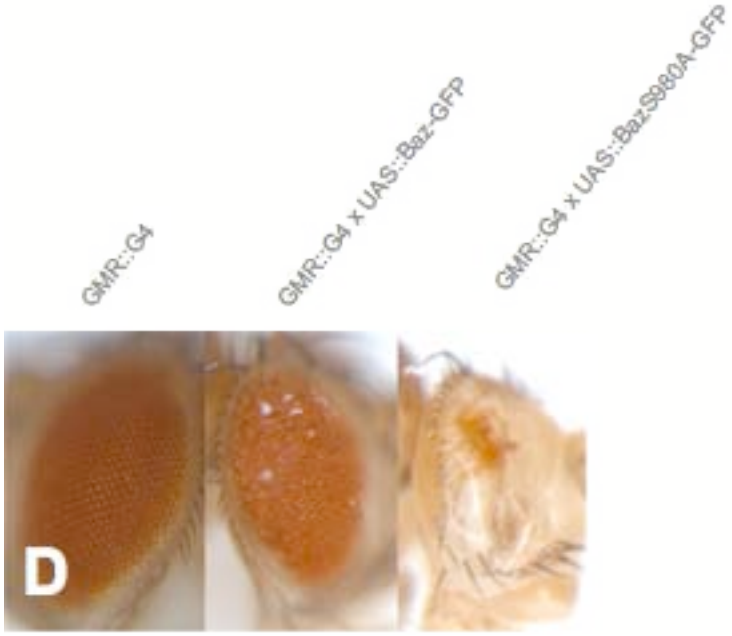

GMR::G4
GMR::G4 x UAS::Baz-GFP
GMR::G4 x UAS::BazS980A-GFP
D

Figure 5

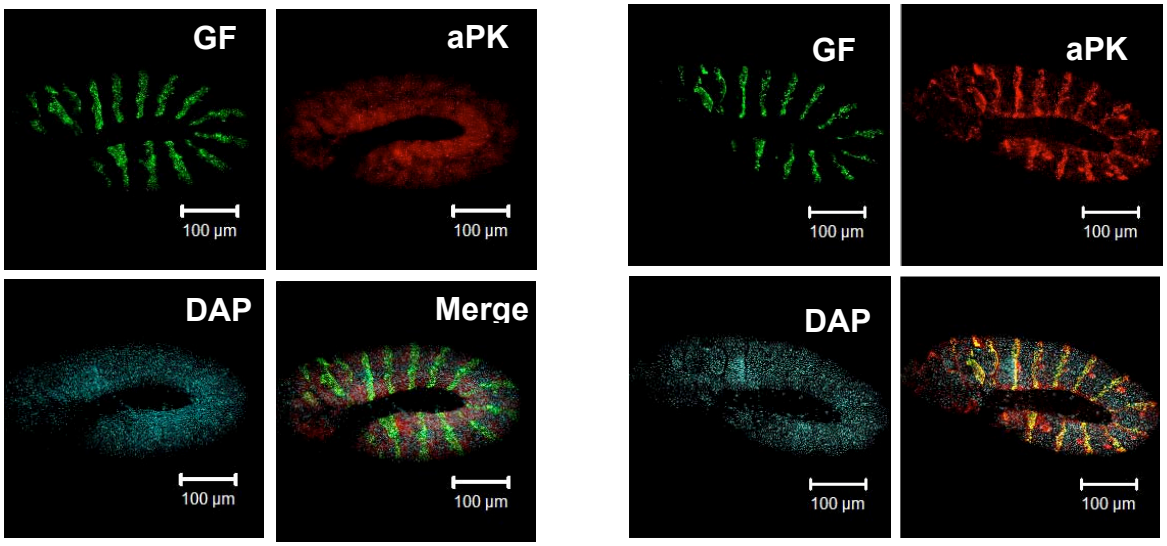
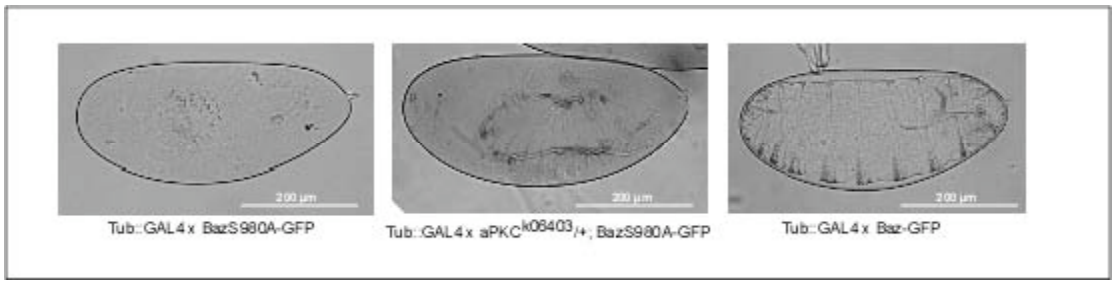

GF
aPK
DAP
Merge
100 µm
A
en::G4 x UAS::BazΔPDZ1-3
B
en::G4 x UAS::BazΔPDZ1-3S980A

Figure 6


Tub::GAL4 x BazS980A-GFP
Tub::GAL4 x aPKC$^{k06403}$/+; BazS980A-GFP
Tub::GAL4 x Baz-GFP

# 5. Discussion

## 5.1. Implications of the structural analysis of the Bazooka protein

Within this study, a systematic structure-function analysis of the Baz protein in different polarized cell types in *Drosophila* was performed, showing that the membrane association of Baz is facilitated by a new lipid binding motif located at the C-terminus of the protein, whereas the N-terminal, highly conserved domains are not crucial for membrane association (see 3.1). Furthermore, this study showed that the subcellular localization of Baz is differentially regulated in the context of the cell type. This is important, as although basic mechanisms for the regulation of cell polarity are conserved between different polarized cell types, they are apparently modulated in a tissue-specific manner. One example is the difference between the ectodermal epithelium (e.g. in the embryonic epidermis) and the NBs. Whereas the N-terminal conserved region 1 (CR1) is dispensable for proper localization of Baz to the ZA in the epidermis, it is required for localization to the apical cortex in NBs.

In complementation to the study presented in 3.1, it would be very interesting to see how the mutant proteins described in 3.1 are localized in a *baz*-negative background. Furthermore, it would be important to know whether the C-terminal truncation constructs (BazΔ1096-1464, BazΔ1222-1464, BazΔ1325-1464, BazΔ1461-1464), BazΔPDZ3 and BazΔ1096-1464PHP, which all are able to rescue the embryonic lethality of *baz* zygotic mutants (cp. 3.1), can also rescue maternally and paternally mutant embryos and whether they can compensate for the loss of *baz* function in germ-line clone egg chambers. Particularly for BazΔ1096-1464 we doubt whether it would rescue *baz* germ line clone mutants, because it is only to a minor extent correctly localized at the membrane and shows a significant cytosolic staining. However, also BazΔ1461-1464 and BazΔ1096-1464PHP are of great interest, as both localize normally at the apical junctional region, but a potential PDZ-domain consisting of the last four amino acids of Baz is deleted. In late embryonic, larval and pupal development this domain appears to be dispensable, but perhaps it plays a crucial role in oogenesis or in the establishment of the first junctional complexes during cellularization in the early embryo. In these stages, the maternal component is still present in zygotic *baz* mutants. Therefore, experiments are under way in which insertions of distinct GFP-Baz deletion constructs inserted on the third chromosome

are recombined with a ubiquitous driver line (da::GAL4, cp. 3.1) and crossed to a *baz* allele carrying an FRT site ($Baz^{815-8}$, FRT19A, (McKim et al., 1996)). Using the FLP-FRT technique (Golic and Lindquist, 1989), female egg chambers can be generated which lack maternal Baz protein but carry the mutant GFP-tagged protein.

One important open question regarding the structure and function of the Baz protein is how it is posttranslationally processed: As can be seen by Western blotting with either GFP antibody using N-terminally GFP-tagged Baz constructs or with an antibody raised against the N-terminal 317aa (Wodarz et al., 1999), several bands of different size are detected corresponding to N-terminal fragments of Baz (cp. 3.2 Figure 1 and Appendix 6.2). In contrast, using an antibody recognizing the last 15 amino acids, only one band representing full length Baz is detectable (data not shown). As these bands are stable and reproducible and definitely more predominant than the band corresponding to full length Baz, they are presumably not only degradation products but rather the result of specific cleavage steps. This raises several questions: first of all, what is the function of the distinct cleavage products? Second, which proteases are involved in the processing of full length Baz? And third, where and in which context Baz is cleaved: directly after the translation event in the cytosol or after membrane recruitment, within the PAR/aPKC complex or before association with its binding partners.

Some ideas can be derived from the structural analysis (cp. 3.1 and Appendix 6.2): As the GFP fluorochrom which is N-terminally attached to full length Baz is exclusively detectable at the membrane and never in other subcellular compartments e.g. the nucleus, the cytosol or vesicular structures, it is clear that either the GFP itself is cleaved and rapidly degraded or the most N-terminal region is always associated with the membrane. This is further supported by the observation that the signal detected by indirect immunofluorescence with the antibody raised against the N-terminal region is always cortical, too. In contrast, truncation of Baz at amino acid 968 (BazΔ969-1464), directly N-terminal of the aPKC-binding domain, results in cytosolic localization of the mutant protein. However, multiple fragments observed in Western Blot with the N-terminal or GFP antibody are much smaller than the calculated size of BazΔ969-1464 (Appendix 6.2). This would mean that the proteins corresponding to these bands should be detectable in the cytoplasm if they are not linked to the membrane by a

different mechanism, which is rather unlikely, as the same cleavage pattern of smaller bands is also detected in BazΔ969-1464, which localizes to the cytosol. Therefore it remains elusive, where the forms of Baz corresponding to these smaller bands are localized in vivo.

Regarding the fate and potential function of the C-terminal cleavage products corresponding to the N-terminal fragments we know virtually nothing – either they are degraded or they are targeted to specific compartments, fulfilling distinct functions. Interestingly, an antibody raised against the peptide Baz973-985 phosphorylated at serine 980 (cp. 3.2), shows besides a weak membrane staining a strong nuclear staining. This suggests that a fragment containing this peptide localizes to the nucleus. This is even more exciting because we found several nuclear proteins as potential interaction partners of Baz in a yeast-two-hybrid screen. For mammalian PAR-3, nuclear localization and a potential function in DNA-damage repair pathways has been described previously (Fang et al., 2007). In their experiments, the authors used an antibody raised against a C-terminal GST-fusion protein of PAR-3 and they were able to detect PAR-3 in the nucleus. With the peptide-antibody raised against the last 15aa of Baz, we only detect a cortical staining, indicating that all fragments containing these aa are not localized to the nucleus.

Taken together there are still many open questions regarding the generation, regulation and function of different fragments of the Baz protein. In particular, the potential function of Baz in the nucleus in the control of chromatin structure and transcription will be in the focus of future research in this field.

## 5.2. Phosphorylation of Bazooka: Only two pieces of a great puzzle

In the second part of this work, two important phosphorylation events have been investigated: First, the role of phosphorylation of serine 1085 by PAR-1 and subsequent dephosphorylation by PP2A in the regulation of apical-basal polarity in dividing NBs. Second, the phosphorylation of serine 980 by aPKC, which is crucial for the establishment of cell polarity in ectodermal epithelial cells. Both studies demonstrate that the posttranslational modification of Baz is necessary and important for the physiological function of the protein. Therefore, an exactly tuned regulation of phosphorylation and dephosphorylation events must take place to ensure proper subcellular localization of Baz in the context of different cell types. One mechanism to restrict such protein modifications to specific subcellular compartments is the targeting of the particular kinases or phosphatases to these compartments. This is the case for PAR-1, which is restricted to the basolateral membrane in epithelial cells and to the basal cortex in dividing NBs (cp 3.2). The restricition of PAR-1 to these membrane domains is presumably achieved by an active exclusion pf PAR-1 from the apical membrane domain by aPKC. In mammalian cells, aPKC phosphorylates PAR-1, which subsequently dissociates from the membrane (Hurov et al., 2004; Suzuki et al., 2004).

However, many if not most kinases and phosphatases are not specifically targeted to a distinct subcellular localization but are rather diffusely distributed in the cytoplasm and the nucleus, for example Protein phosphatase 1, PP2A and LKB1/PAR-4 (Bonaccorsi et al., 2007; Janssens and Goris, 2001; Jones et al., 1995). Therefore another possibility to spatially restrict kinase / phosphatase activity is to establish phosphorylation cascades, leading to the activation or inactivation of proteins and kinases. Apart from the mentioned aPKC-PAR-1 interaction, the binding of the 14-3-3 proteins to Baz/PAR-3 is another example: The ubiquitously expressed 14-3-3 proteins are only bound after phosphorylation of their target sequences (Hurd et al., 2003), in the case of Baz after phosphorylation of serine 151 and 1085 by PAR-1. As this occurs only at the basal cortex in NBs, where PAR-1 is expressed, the activities of 14-3-3 proteins are also restricted to that specific localization (see also 3.2).

In this study we described two different phosphorylation events of Baz with dramatic effects on the regulation of cell polarity. However, we have obtained evidence that there exist far more phosphorylation sites in the Baz protein: Treatment of cells transfected with Baz-GFP with high concentrations of phosphatase inhibitors and subsequent immunoprecipitation of Baz protein and Western blot against Baz revealed a significant change in the protein mobility in SDS-PAGE by more than 10kDa (cp 3.2, Figure 2). This band-shift may be due to an altered charge of the Baz protein upon modification by phosphate groups. As the three described phosphorylation sites/events do not lead to such a dramatic increase in the band size, it is clear that Baz is phosphorylated at additional residues. Indeed, two proteome-wide screens for phosphorylated proteins in *Drosophila* identified several phosphorylated amino acids in Baz: In the database Phospho Pep, phosphorylated peptides isolated from *Drosophila* Kc167 cells identified by mass spectrometry were assigned to proteins (Bodenmiller et al., 2008). In this study, 14 serine residues and two threonine residues of Baz were found to be phosphorylated. Interestingly, of the three previously described phosphorylation sites only S151 was found in this screen, indicating that by this approach not all phosphorylated peptides were detected. This implies that Baz may be phosphorylated at more than 20 different amino acid residues. A second study used sodium orthovanadate as a specific inhibitor for tyrosine phosphatases to enhance tyrosine phosphorylation of proteins in *Drosophila* S2 cells. After precipitation of proteins containing phosphorylated tyrosines with an anti phospho-tyrosine antibody, proteins were digested and peptides analyzed by mass spectrometry (Chang et al., 2008). For Baz, four peptides carrying phosphorylated tyrosines were found. Interestingly, the mammalian homolog PAR-3 is tyrosine phosphorylated at residue 1127 in an epidermal growth factor dependent manner (Wang et al., 2006). Although Y1127 is not conserved in Baz, these two publications demonstrate that Baz might be tyrosine phosphorylated, too. However, we failed to detect tyrosine phosphorylation of Baz immunoprecipitated from cell lysates in Western blotting (data not shown). One reason might be that the phosphorylation is rather transient and not stable enough to be detected in Western blotting. Nevertheless the discussed findings demonstrate that we are far from understanding the entire phosphorylation repertoire of the Baz protein and especially how it is regulated in the context of Baz's function in the establishment of cell polarity. To obtain more information about additional phosphorylation sites, the corresponding

kinases/phosphatases and the role of the particular phosphorylation events in the context of cell polarity will be one of the future goals of our research.

One kinase of particular interest is LKB1, the *Drosophila* homologue of *C.elegans* PAR-4. LKB1 was originally identified in C.elegans in a screen for embryonic asymmetry (Watts et al., 2000). This function is conserved in the *Drosophila* oocyte, where LKB1 functions in the establishment of anterior-posterior axis formation (Martin and St Johnston, 2003). Various studies in mammalian cells indicate that LKB1 is also involved in the regulation of epithelial cell polarity and functions as a tumor suppressor protein (for review see (Baas et al., 2004).

In mammalian cells, LKB1 phosphorylates PAR-1 in the activation loop of the kinase domain, resulting in activation of PAR-1 (Lizcano et al., 2004). Martin and St. Johnston obtained contradictory results and showed that in *Drosophila* LKB1 acts genetically downstream of PAR-1 and is itself phosphorylated by PAR-1 (Martin and St Johnston, 2003). It is also possible that LKB1 phosphorylates not only PAR-1 but also other members of the PAR family, e.g. Baz, PAR-6 or aPKC which would be another intriguing piece in the network of PAR-proteins regulating cell polarity. In *Drosophila* NBs, LKB1 has been shown to be crucial not only for asymmetric cell division and spindle formation, but also for correct localization of the PAR/aPKC complex to the apical and Mira to the basal cortex (Bonaccorsi et al., 2007). However, in this context, no phosphorylation target has been identified yet. Therefore, components of the PAR/aPKC complex would be ideal targets of LKB1, particularly because their localization is disturbed whereas Pins and Gαi are localized normally. These proteins have been shown to act in a parallel pathway which functions redundantly to the PAR/aPKC complex in asymmetric division and spindle orientation (Cai et al., 2003; Fuse et al., 2003; Izumi et al., 2004; Yu et al., 2003). The penetrance of spindle orientation and division defects is increased in double mutant for LKB1 and Pins compared to the according single mutants, indicating that they act in parallel pathways, which suggests that LKB1 may be involved in the regulation of the PAR/aPKC complex.

Another candidate for a Baz phosphorylating kinase is LK6, a serine-threonine kinase, which was found to interact with the Baz N-terminal region in the same yeast-two-hybrid screen as PP2A (cp. 3.2, Egger-Adam PhD thesis 2006). This is of great interest, because LK6 associates with microtubules and localizes to the centrosomes

(Kidd and Raff, 1997), which might provide a direct link between cortical Baz and the formation and orientation of the mitotic spindle, which has up to now only been shown by genetic interactions (Wodarz, 2005).

On the other hand, regarding additional phosphatases implicated in Baz dephosphorylation, protein phosphatase 1 (PP1) is one of the most likely candidates. Besides PP2A, PP1 is the second major, ubiquitously expressed phosphatase and it has already been demonstrated to dephosphorylate mouse PAR-3 (Traweger et al., 2008). Interestingly, in that study the serine residues corresponding to serine 151, 980 and 1085 were described to be dephosphorylated by PP1. Furthermore, PP1 binds to several fragments of PAR-3, including the PDZ domains, which indicates that there might be more phosphorylation sites affected by PP1. As from our experiments we conclude that PP2A dephosphorylates serine 1085 (see 3.2) there is either a redundant mechanism or in contrast to the phosphorylation event, the dephosphorylation of serine 1085 is not conserved throughout evolution. Hoewever, it also has to be considered whether the reported in vito dephosphorylation of all three reported phosphorylation sites really takes place in vivo and if so, whether this is of physiological relevance. Nevertheless PP1 might dephosphorylate distinct serine/threonine residues of Baz and thereby it may take part in the complex regulation of Baz in the context of cell polarity.
Apart from PP1, several other phosphatases must be taken into account with respect to Baz dephosphorylation. One example is protein phosphatase 4, whose regulatory subunit Falafel associates with Mira in larval NBs (Sousa-Nunes et al., 2009). Nuclear Falafel prevents Pros from entering the nucleus in the NB and knock-down of *falafel* results in dissociation of Mira from the cortex, indicating that Falafel plays a crucial role in the establishment of apical-basal polarity of dividing NBs and thereby in the asymmetric cell division. However, direct dephosphorylation of Mira or other proteins by Falafel has not been demonstrated yet.

Apart from phosphorylation, other posttranslational modifications of Baz might regulate its localization, its affinity to binding partners etc. For example, attachment of ubiquitin (mono- or polyubiquitination) emerges more and more to be not only important for the targeting to the proteasome and subsequent degradation of a protein, but can also function to modify localization or function of a protein (Sun and Chen,

2004). One example in NBs is Mira, which has been found to be ubiquitinylated at its C-terminal region. Removal of this domain results in mislocalization of Mir to the cytosol, whereas replacement of this domain by ubiquitin restores the physiological localization (Slack et al., 2007). Blotting of precipitated Baz protein reveals that several cleavage products of Baz are ubiquitinylated (data not shown), but it remains elusive, whether this leads to protein degradation or whether there is any additional functional relevance in the attachment of ubiquitin molecules to Baz.

A mechanism similar to ubiquitinylation is SUMOylation. Here, a small molecule called SUMO (small ubiquitin-homologous modifier) is attached to lysine residues of the protein, preferentially at sites matching a consensus motif (hydrophobic – K – X – D/E). Similar to ubiquitinylation, SUMOylation has been reported in several contexts to regulate protein localization and activity, especially in transcriptional regulation (Perry et al., 2008; Talamillo et al., 2008). Baz contains several potential SUMOylation sites (predicted by SUMOPlotTM, http://www.abgent.com/tools/sumoplot). However, according to Western blots with an anti-SUMO antibody on precipitated Baz protein, Baz does not appear to be SUMOylated (data not shown).

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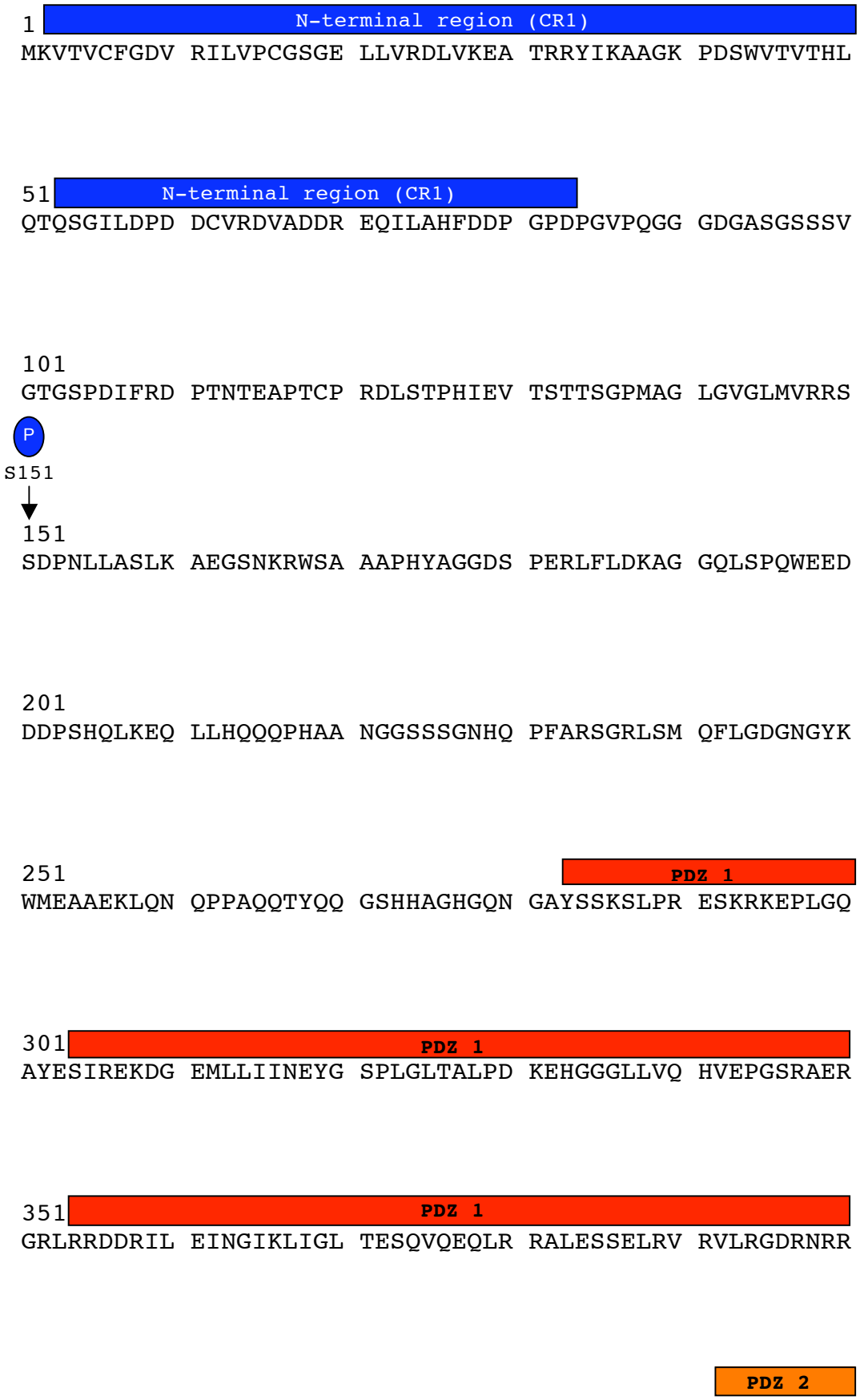
# 7. Appendix

## 7.1. Abbreviations

| | |
|---|---|
| aa | amino acids |
| aPKC | atypical protein kinase C |
| Baz | Bazooka |
| Crb | Crumbs |
| Dlg | Discs large |
| GαI | G-protein αi |
| GFP | green fluorescent protein |
| GMC | ganglion mother cell |
| Insc | Inscuteable |
| kDa | kilodalton |
| Lgl | lethal giant larvae |
| Mira | Miranda |
| NB | neuroblast |
| PAR | partitioning-defective |
| Pins | Partner of Inscuteable |
| PP1 | protein phosphatase 1 |
| PP2A | protein phosphatase 2A |
| SAR | sub-apical region |
| TJ | tight junction |
| ZA | zonula adherens |

## 7.2. Bazooka sequence



401
QQRDSKVAEM VEVATVSPTR KPHAAPVGTS LQVANTRKLG RKIEIMLKKG

451 PDZ 2
PNGLGFSVTT RDNPAGGHCP IYIKNILPRG AAIEDGRLKP GDRLLEVDGT

501 PDZ 2
PMTGKTQTDV VAILRGMPAG ATVRIVVSRQ QELAEQADQP AEKSAGVAVA

551
PSVAPPAVPA AAAPAPPIPV QKSSSARSLF THQQQSQLNE SQHFIDAGSE

601
SAASNDSLPP SSNSWHSREE LTLHIPVHDT EKAGLGVSVK GKTCSNLNAS

651 PDZ 3
GSSASSGSNG LMKHDGDLGI FVKNVIHGGA ASRDGRLRMN DQLLSVNGVS

701 PDZ 3
LRGQNNAEAM ETLRRAMVNT PGKHPGTITL LVGRKILRSA SSSDILDHSN

751
SHSHSHSNSS GGSNSNGSGN NNNSSSNASD NSGATVIYLS PEKREQRCNG

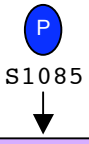
801
GGGGGSAGNE MNRWSNPVLD RLTGGICSSN SAQPSSQQSH QQQPHPSQQQ

851
QQQRRLPAAP VCSSAALRNE SYYMATNDNW SPAQMHLMTA HGNTALLIED

901
DAEPMSPTLP ARPHDGQHCN TSSANPSQNL AVGNQGPPIN TVPGTPSTSS


951 aPKC-binding
NFDATYSSQL SLETNSGVEH FSRDALGRRS ISEKHHAALD ARETGTYQRN

1001
KKLREERERE RRIQLTKSAV YGGSIESLTA RIASANAQFS GYKHAKTASS




1051 CR2
IEQRETQQQL AAAEAEARDQ LGDLGPSLGM KKSSSLESLQ TMVQELQMSD

1101 CR3
EPRGHQALRA PRGRGREDSL RAAVVSEPDA SKPRKTWLLE DGDHEGGFAS

1151
QRNGPFQSSL NDGKHGCKSS RAKKPSILRG IGHMFRFGKN RKDGVVPVDN

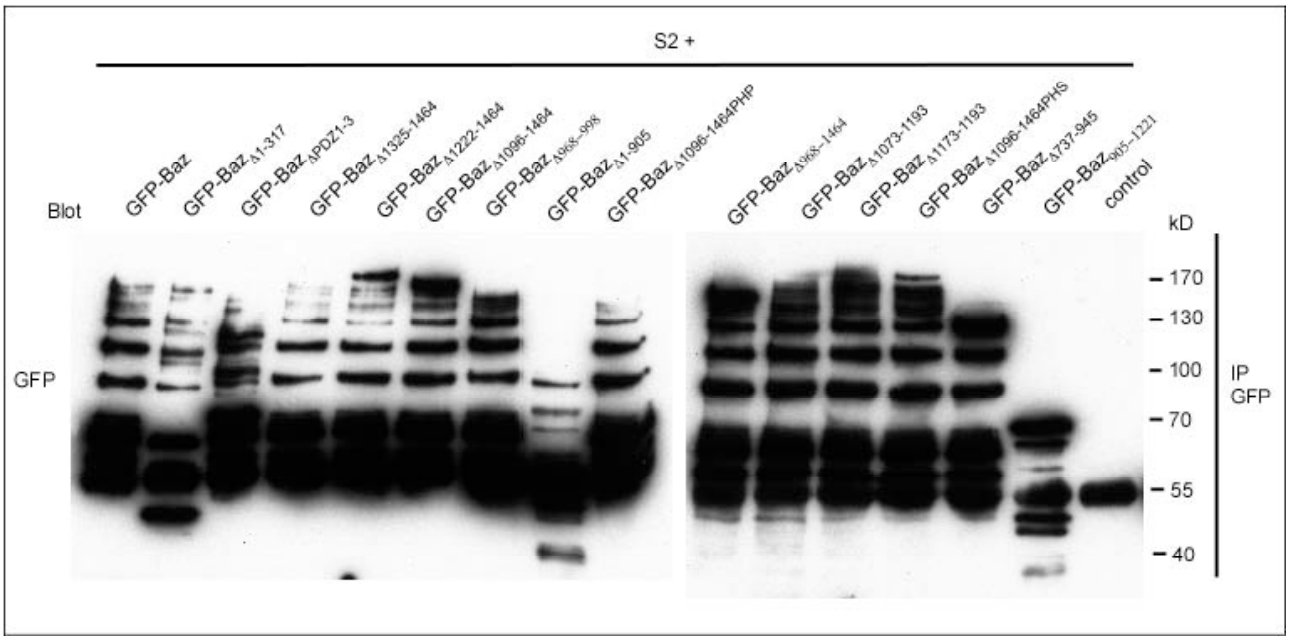
1201
YAVNISPPTS VVSTATSPQL QQQQQQQLQQ HQQQQQIPTA ALAALERNGK

1251
PPAYQPPPPL PAPNGVGSNG IHQNDIFNHR YQHYANYEDL HQQHQQHQIS

```
1301
RRHQHYHSQR SARSQDVSMH STSSGSQPGS LAQPQAQSNG VRPMSSYYEY

1351
ETVQQQRVGS IKHSHSSSAT SSSSSPINVP HWKAAAMNGY SPASLNSSAR

1401
SRGPFVTQVT IREQSSGGIP AHLLQQHQQQ QLQQQQPTYQ TVQKMSGPSQ

1451        PDZ-B
YGSAAGSQPH ASKV
```

Baz protein sequence (isoform A). The conserved domains are marked with bars (the up to now unannotated conserved regions aa 1073-1093 and 1173-1193 are annotated as CR2 and CR3). The three reported phosphorylation sites are indicated.

## 7.3. Western Blot of Baz constructs




S2R cells were transfected with Baz expression constructs, which were N-terminally GFP-tagged. GFP-Baz proteins were immunoprecipitated using an anti-GFP antibody (see 3.2 for details), proteins were resolved on 10% SDS-PAGE and blotted against GFP.

# 8. Curriculum vitae

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Germany

08.12.1980 : Born in Muenster (Germany)

1987-1990 : Visit of the primary school in Everswinkel

1990-2000 : Visit of the grammar school in Warendorf, graduation with the "Abitur"

2000-2006 : Study of veterinary medicine at the School of Veterinary Medicine, Hannover, Graduation in 2006

2003 : Participation in the Cornell Leadership Program for Veterinary Students

2003-2006 : Experimental PhD work in the Institute of Physiological Chemistry : „Expression and characterization of Protocadherin LKC in mammalian cells – an new potential growth regulator in epithelial cells", graduation as "Dr. med vet." in Mai 2006

2006-2009 : PostDoc position in the Department of Stem Cell Biology, University of Goettingen

**Posters and Presentations :**

- Poster at the meeting Horizons in Molecular Biology (12.09.-15.09.2007):
  1.) Krahn and Wodarz : **„Functional interaction of Protein Phosphatase 2A with Bazooka/PAR-3 during *Drosophila* development"**
  2.) Trierweiler, Krahn and Wodarz : „***Drosophila* as a model organism to study the physiological role of Dymeclin in context with the Dyggve-Melchior-Clausen Dysplasia"**
  3.) Neugebauer, Krahn and Wodarz : „**Analysis of the Function of CG31534 during epithelial development in *Drosophila"***

- Poster at the annual meeting of the German Society for Cell Biology (12.-15.03.2008):
  Krahn and Wodarz : „**Dephosphorylation of Bazooka by PP2A is required for proper apical-basal polarity in embryonic neural stem cells"**

- Presentation at the International Drosophila Research Conference (San Diego, 02.-06.04.2008) Krahn and Wodarz : „**Dephosphorylation of Bazooka by**

**PP2A is required for proper apical-basal polarity in *Drosophila* embryonic neuroblasts"**

- Poster at the meeting Horizons in Molecular Biology (10.09.-13.09.2008): Hogl, Krahn and Wodarz : „**Proper phosphorylation of Bazooka is required for maintenance of epithelial polarity in Drosophila melanogaster"**

- Poster at the meeting of the Germany Society for Developmental Biology (25.-28.03.2009):
  1.) Beati, Krahn and Wodarz : "**The role of the Bazooka-CG31534 interaction for the establishment of cell polarity in Drosophila melanogaster"**
  2.) Krahn et al. : "**Imperfect conservation from fly to man : The C-terminal region of Bazooka but not of PAR-3 facilitates membrane binding**"

- Presentation at the meeting of the Germany Society for Developmental Biology (25.-28.03.2009): Krahn and Wodarz : „**PP2A antagonizes phosphorylation of Bazooka by PAR-1 to control apical-basal polarity in dividing embryonic neuroblasts"**