Dynamic interactions between PAR-aPKC complex and Crb complex in *Drosophila* epithelial cells

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Summary

Epithelial cell polarity is one of the key prerequisites for the establishment of multicellular organisms. The apical-basal polarity of epithelia is controlled by evolutionarily conserved complexes which determine an apical versus a basolateral domain. The PAR-aPKC complex (Baz/PAR-3-PAR-6-aPKC) defines together with the Crumbs complex (Crb-Sdt/Pals1-PATJ) the apical plasma membrane domain of the cell. Interestingly, both complexes are highly dynamic during development. The activity and stability of these two complexes are counterbalanced by basolateral protein complexes, mainly the Scribble-Lethal giant larvae-Discs large complex.

In this study, the apical targeting mechanism of PATJ, which has been described to form a complex with Crb-Sdt, was investigated in detail. In the embryonic epidermis of *Drosophila*, the PATJ-Sdt heterodimer is not initially recruited to the apical cell-cell contacts by binding to Crb but depending on forming a new complex with Bazooka (Baz). The Baz-Sdt-PATJ complex together with Crb-Sdt-PATJ complex co-exist in the mature epithelium of the embryonic epidermis, and might play a redundant role in myosin activity modulation.
As a core component of the PAR-aPKC complex, PAR-6 was shown to regulate the stability of the Crb complex. Indeed, from a loss-of-function study, we found that PAR-6 promoted the stability of the Crb-adaptor protein Sdt, thereby regulating the Crb complex assembly. Further data showed that PAR-6 associated with the proteasomal receptor Rpn13 and prevented the degradation of Sdt indirectly. Importantly, the Sdt degradation phenotype by loss of PAR-6 could be rescued to some extent by downregulation of Rpn13. Thus, we identified a new mechanism of PAR-6 inhibiting proteasomal degradation of Sdt to maintain proper apical-basal polarity.

Lastly, a structure-function analysis of the Crb-adaptor protein Sdt was performed and the localization and function of the mutant proteins in the embryonic epidermis were evaluated. Our *in vivo* data confirmed that the PDZ domain of Sdt is crucial for Crb binding and localization. Interestingly, the conserved N-terminal regions (ECR1 and ECR2) are not crucial for epithelial polarity. In contrast, the GUK domain plays an important role in epithelial polarity, which is independent of Crb stabilization, and the L27N domain is essential for epithelial polarization independently of PATJ binding.

Introduction

Cell polarity is the fundamental characteristic of cells and is featured by the asymmetric distributions of cellular components, including lipids, proteins or RNAs. The asymmetric organization produces distinct cellular functions. Polarized cells such as neurons can propagate the electric signals from dendrites to axons while migrating cells sense the extrinsic chemical signals and migrate in the defined directions. Besides, one type of tissues exhibiting the extreme polarity is the epithelial tissue, which consists of specialized cells that tightly interconnect with each other and form single or multiple layered epithelia.

1.1 Cell polarity models in *Drosophila*

Over thirty-year work proved the fruit fly *Drosophila melanogaster* to be an excellent model to study fundamental biological issues due to a vast number of state-of-art genetic approaches and the relatively short life cycle. In contrast to mammalian cell culture which only focuses on a certain amount of cell types or mouse model which is time-consuming and has many ethical issues, *Drosophila* allows us to answer questions with *in vivo* data: not only focusing on one cell type or one gene but also having a whole picture on the entire organism.

Up to date, there are many models developed for cell polarity studies.

1. Egg chamber. *Drosophila* egg chamber is a versatile system to study many different polarity issues. A typical egg chamber consists of the monolayer epithelia organized by follicular cells, germ cell derived nursing cells and one oocyte. Follicular cells are polarized cells with apical facing towards inside and basal facing to outside of the chamber. They are the largely used model to study epithelial polarity and planar cell polarity. Oocyte has an anterior-posterior axis (from nursing cells' side to the next egg chamber).

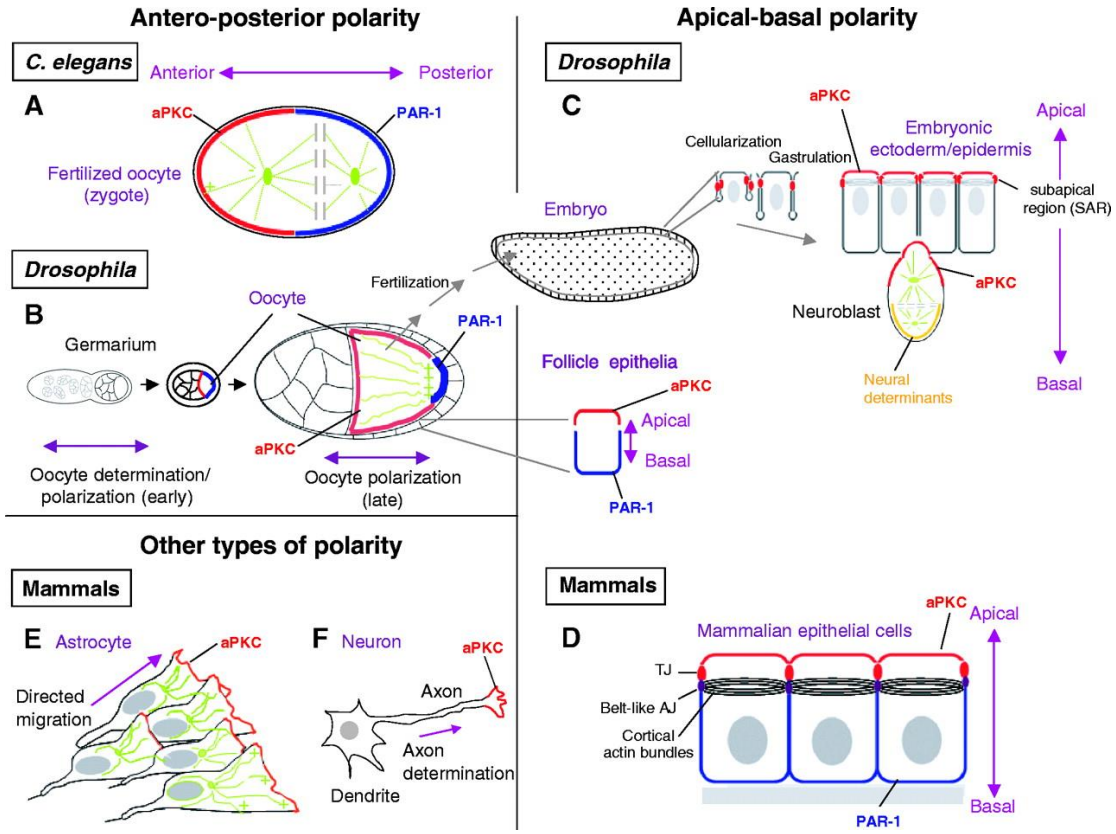

Figure 1.1 Various types of cell polarity (Suzuki and Ohno 2006).

2. Ectodermal epithelia. Ectodermal epithelia can be subdivided into embryonic epidermis, gut and trachea systems. Embryonic epidermis provides an excellent model to study the *de novo* epithelial polarity establishment, polarity maintenance as well as complicated morphogenesis.

3. Imaginal discs. Imaginal discs are sac-like epithelial structure found inside in larva. Along with the metamorphosis, imaginal discs turn into the external structure of many body parts of *Drosophila*. There are 19 discs in total. However, only the wing discs and eye discs are widely used as an epithelial model due to their relatively large size. They are nice models to study PCP and many different cross-talking pathways of polarity proteins, such as Notch signaling and Wnt pathway.

4. Other polarized models. Neural stem cells (Neuroblasts) are a great model to study the stem cell biology, especially focusing on the asymmetric cell division related issues. They start to massively proliferate from embryonic stage nine. Photoreceptor

cells are also polarized and appropriate for polarity studies.

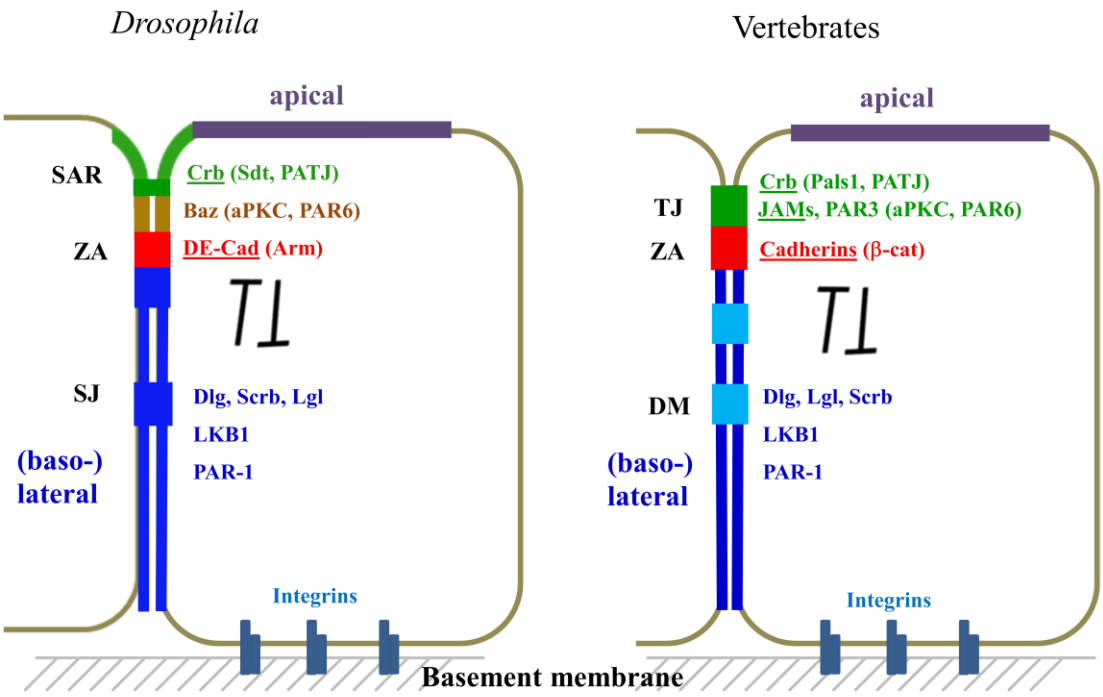

Figure 1.2 Epithelial polarity is highly conserved from *Drosophila* to vertebrates. Epithelial polarity is regulated by various evolutionarily conserved protein complexes. At the apical region, there are apical polarity regulators (APR), namely the Baz/PAR-3-aPKC complex and the Crb complex. The basolateral membrane is marked by the basolateral polarity regulators (BLPR), such as the Scrb-Dlg-Lgl complex. APR and BLPR are counterbalancing each other to ensure the integrity of cell polarity.

1.2 Epithelial cell polarity

Polarized epithelia outline the cavities and surfaces of the body of animals, functioning as the selective and physical barrier between the tissue compartments or between environment and inner space (Muthuswamy and Xue 2012; Tepass 2012). To achieve this function, epithelial cells are highly polarized and featured by the differentiation of plasma membranes, namely the apical membrane, the lateral membrane and the basal membrane (Laprise and Tepass 2011; Thompson 2013). The apical membrane faces towards the outside environment or luminal space of internal

organs, while the lateral membrane connects adjacent cells within the epithelium and the basal membrane is linked to the basement membrane and further to the connective tissues. The lateral and basal membranes are often summarized as the basolateral domain.

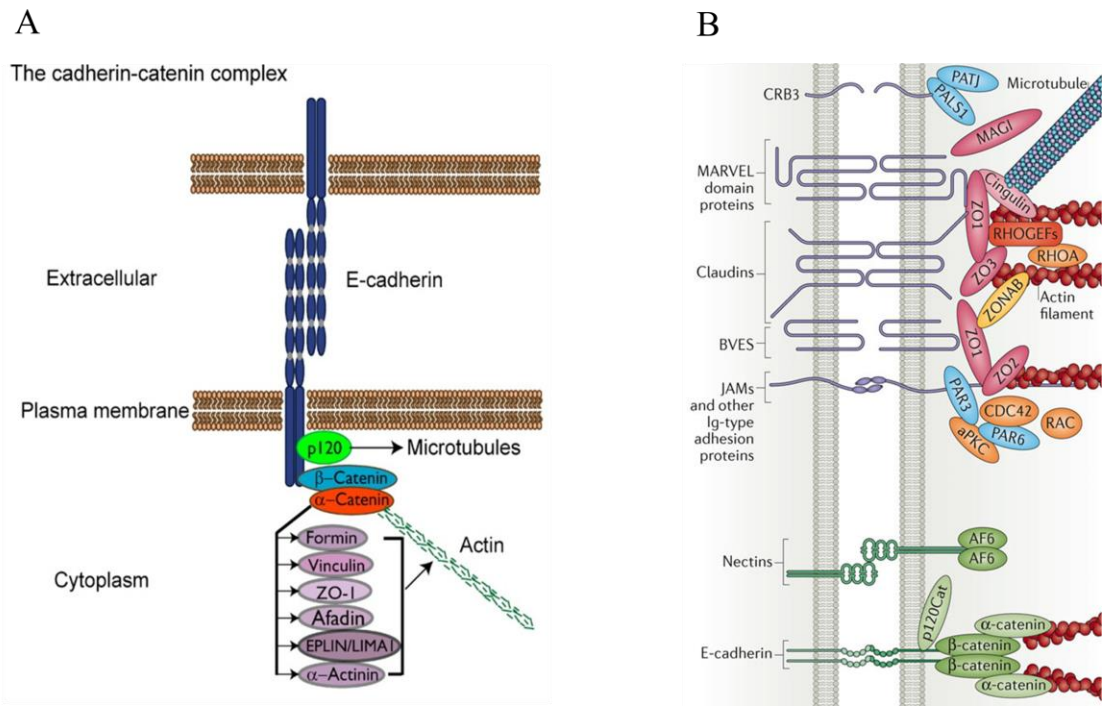

Figure 1.3 Schemes of adherens junctions and tight junctions. (A) E-Cadherin undergoes trans-homophilic interactions to form Cadherin clusters. The E-Cadherin intracellular domain contains binding sites for the catenins p120 and β-catenin. β-catenin binds α-catenin, which in turn binds actin and several actin-associated proteins (Baum and Georgiou 2011). (B) A brief overview of types of tight junction proteins with only representatives of the main groups shown. For details, check review (Zihni, Mills et al. 2016).

Apical-basal axis establishment and maintenance require cell-cell contacts and junction formation. The distinct apical and basolateral membrane domains are separated by the belt-like structure, called *zonula adherens* (ZA). The ZA (also known as adherens junctions, AJs) consists of Cadherin-Catenin complexes and Actin filaments (F-actin) (Baum and Georgiou 2011; Takeichi 2014). Specifically, the extracellular domain of E-Cadherin can dimerize with trans-homophilic interactions to establish Cadherin clusters while the intracellular domain contains binding sites for

the Catenins p120 and β-catenin, thereby forming the Cadherin–Catenin complex. β-catenin binds α-catenin, which in turn binds F-actin directly or indirectly via several actin-associated proteins, including α-actinin, Vinculin, Afadin and Formin-1 (Fig. 1.3 A). A bundle of F-actin runs in parallel to cell borders and provides the strength to single cells gathering all of them into the epithelium. The maturation of this structure provides a barrier which is necessary for the differentiation of distinct apical and basolateral domains. Below the ZA, cadherin proteins can also be detected throughout the lateral membranes, however, with a rather low intensity in these regions (Takeichi 2014).

Apical to the ZA, Tight Junctions (TJs) are formed between adjacent epithelial cells in vertebrates, functioning as the main paracellular diffusion barrier and controlling the diffusion of ions and solutes (Anderson and Van Itallie 2009; Zihni, Mills et al. 2016). Electron microscopy revealed that TJs form close focal contacts between two neighboring cells leaving pores of different sizes for water and ions in between (Zihni, Mills et al. 2016). Many TJ associated transmembrane proteins are identified from intensive molecular studies. Transmembrane proteins include claudins which are considered to be most critical for defining TJ functionality, junctional adhesion molecules (JAMs), Occludin and Crumbs3 (Crb3). Similar to Cadherins, these TJ-associated proteins also exhibit a homophilic interaction pattern while their intracellular adaptors are highly diverse and dynamic (Fig. 1.3 B). In *Drosophila* epithelium, instead of forming TJs, the corresponding region (named as the subapical region, SAR) is featured by enrichment of the single pass transmembrane protein Crb (Bulgakova and Knust 2009). However, the function of Crb homophilic interaction is rather obscure. The paracellular barrier function which is accomplished by TJ in vertebrates is largely achieved by formation of a *Drosophila*-specific junction called Septate Junction (SJ). SJs localize basally to the ZA and contain many evolutionarily conserved proteins, like Claudin related proteins, e.g. megatrachea, Kune-kune and Sinuous (Behr, Riedel et al. 2003; Genova and Fehon 2003; Wu, Schulte et al. 2004; Wu, Marcus et al. 2007; Nelson, Furuse et al. 2010).

In vertebrates, the basolateral desmosomes (DMs) connect the intermediate filament cytoskeleton with the cell cortex.. DMs are widely found in epithelial tissues of specialized organs or systems which need strong adhesion, such as skin and heart (Garrod and Chidgey 2008). Similar homophilic bindings of the Cadherin family proteins bridge the extracellular spaces and provide platforms to assemble intracellular protein complexes. DMs are not found in *Drosophila* lateral membrane of the epithelial cells because the genes encoding core DM components (such as Desmogleins and Desmocollins, Plakoglobins and Plakophilin) are not conserved in *Drosophila*.

Epithelia are anchored to the basement membrane through the interaction between integrins and extracellular matrix (ECM) (Campbell and Humphries 2011). Integrins are transmembrane receptors, which form heterodimers from different protein isoforms. By linking the cytoskeleton and ECM, integrins allow cells to perceive and response properly to a dynamic microenvironment.

1.3 Epithelial polarity determinants and complexes

Since the 1980s, the genetic screenings in *Caenorhabditis elegans* and *Drosophila* led to identification of a wide range of polarity proteins (Tepass 2012). After intensive studies for around four decades, researchers figure out that multiple distinct and evolutionarily conserved groups of proteins constitute the epithelial cell polarity program, including: the Crb complex, Baz/PAR-3-aPKC complex, and the Lgl-Scrib-Dlg complex.

1.3.1 The Crumbs complex

The canonical Crumbs complex consists of the transmembrane protein Crb (Crb1-3 in mammals), its intracellular adaptor Stardust (Sdt, protein associated with Lin7 1 Pals1/Mpp5 in mammals), Pals1 associated tight junction protein (PATJ) and Lin-7 (Bulgakova and Knust 2009; St Johnston and Ahringer 2010). The formation of the Crumbs complex is achieved by direct interactions among these different proteins.

Sdt/Pals1 acts a hub, on one hand, binding the C-terminal ERLI motif of Crb with its PSD-95/Discs-large/ZO-1 (PDZ) domain and stabilizing Crb at the apical junctional region, the SAR in *Drosophila* (Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001; Li, Wei et al. 2014). On the other hand, PATJ and Lin7 bind to the L27-N and C domains of Sdt/Pals1 respectively (Roh, Makarova et al. 2002; Bachmann, Timmer et al. 2004).

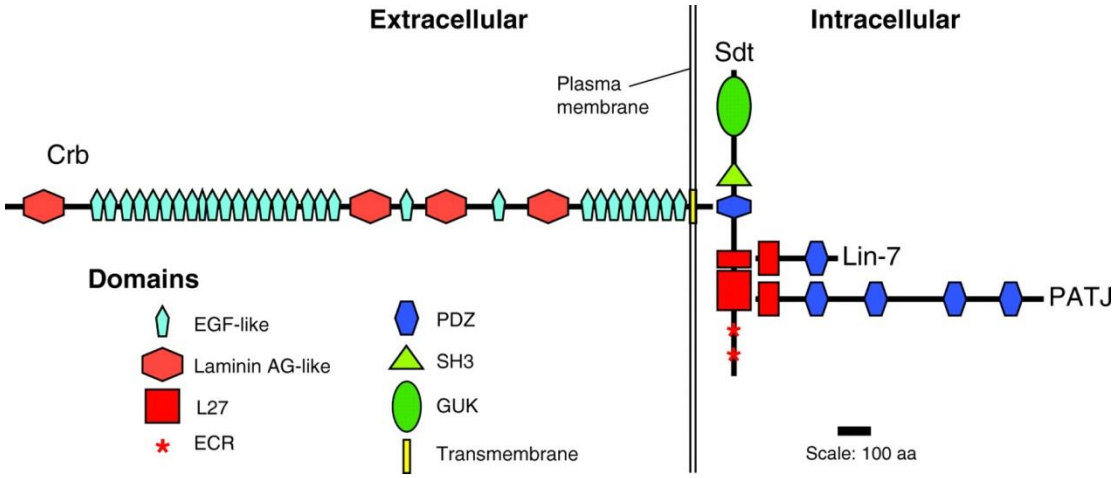

Figure 1.4 Scheme diagram shows the core proteins of the *Drosophila* Crumbs complex. The canonical Crumbs complex consists of the transmembrane protein Crb, its intracellular adaptor Sdt, Pals1 associated tight junction protein PATJ and Lin-7 (Bulgakova and Knust 2009).

1.3.1.1 Crumbs

Crb is a type I transmembrane protein and is conserved from *C. elegans* and *Drosophila* to human (Tepass, Theres et al. 1990; Pocha and Knust 2013). Crb was initially identified as the apical determinant from loss-of–function studies in *Drosophila* epithelia (Tepass, Theres et al. 1990). *Drosophila* Crb has a large extracellular domain composing of 29 epidermal growth factor (EGF) like domains and four laminin A globular-domain like repeats. The intracellular part of Crb which is rather small (only 37 amino acids in total) contains two functionally important and conserved motifs: The C-terminal PDZ binding motif (also designed ERLI motif in *Drosophila* according to the last four amino acids) and 4.1/ezrin/radixin/moesin

(FERM)-binding motif (Tepass, Theres et al. 1990).

Mutation of *crb* abolishes the formation of the apical domain, whereas overexpression increases the apical membrane size at the expense of the basolateral domain (Wodarz, Grawe et al. 1993; Wodarz, Hinz et al. 1995). Similar defects were observed in mammalian systems, for instance in MDCK cells (canine kidney tubular cells) which are widely used as an *in vitro* model for polarity studies (Roh, Fan et al. 2003; Lemmers, Michel et al. 2004). Interestingly, *crb* mutant phenotype can be fully rescued by only expression of the transmembrane domain and the intracellular tail, highlighting the crucial functions of the PDZ-binding motif (mediating binding of the PDZ domain-containing proteins, e.g. Sdt/Pals1) and FERM-binding motif (anchoring Crb to cytoskeleton through FERM containing proteins) (Pocha and Knust 2013). These mechanisms are thought to be very important in inhibiting the endocytosis of Crb (Pocha and Knust 2013).

In contrast, the function of the large extracellular domain in cell polarity regulation is rather obscure. One study indicated that the *trans*-homophilic dimmer formation of *Drosophila* extracellular domains was able to rescue the localization of Crb-extra variant in a *crb* mutant background (Letizia, Ricardo et al. 2013). However, this observation was only found in the boundary between wild-type cells and mutant ones. Nevertheless, the extracellular domain seems to be important in some special cells or under particular conditions during development. It may also be involved in the regulation of several different signaling pathways, such as Notch signaling (Pellikka, Tanentzapf et al. 2002; Muschalik and Knust 2011; Thompson, Pichaud et al. 2013).

1.3.1.2 Stardust/Pals1

Sdt/Pals1 belongs to the membrane-associated guanylate kinase (MAGUK) family, containing two N-terminal evolutionary conserved regions (ECR), two L27 domains, a PDZ domain, a Src homology 3 domain (SH3) and a catalytically inactive GUK domain (Roh, Makarova et al. 2002). Sdt was first identified in a genetic screen in *Drosophila* and loss of *sdt* phenocopies the *crb* mutant (Wieschaus, Nüsslein-Volhard

et al. 1984; Tepass and Knust 1993).

Drosophila sdt employs different splicing strategies, resulting in four different isoforms: Sdt-B, F, H and D (Bulgakova, Rentsch et al. 2010). However, only Sdt-F is expressed throughout the entire embryonic development, while Sdt-B is only expressed at the early stages of embryos (Horne-Badovinac and Bilder 2008). Intriguingly, apart from the apically localized Sdt protein, the *sdt* mRNA also exhibits the apical localization in embryonic epidermis and follicular cells (Horne-Badovinac and Bilder 2008).

Recent structural analysis by crystallization revealed that not only the PDZ domain but also the PDZ-SH3-MAGUK tandem is crucial for Crb binding and *in vitro* cysts formation in an MDCK cell culture model (Li, Wei et al. 2014).

1.3.1.3 PATJ and Lin7

PATJ and Lin7 exhibit an N-terminal L27 domain and subsequent PDZ domains (four in *Drosophila* PATJ and one in *Drosophila* Lin7). PATJ was initially described as discs lost (Dlt) by a mistake (Bhat, Izaddoost et al. 1999). Loss-of-function studies indicate that PATJ seems to be not important for the early embryonic development as evidence that *PATJ* mutant embryos do not show obvious defects in either Crb-Sdt stabilization and localization or junction formation (Pénalva and Mirouse 2012; Sen, Nagy-Zsvér-Vadas et al. 2012; Zhou and Hong 2012). However, PATJ is indeed very important in the photoreceptor cells to stabilize Crb and Sdt at the stalk membrane and prevent strong light-induced degeneration of rhabdomeres (Nam and Choi 2006; Richard, Grawe et al. 2006, Zhou and Hong 2012). Our group has recently shown that PATJ regulates myosin phosphorylation by directly binding to myosin binding subunit (MBS) thereby inhibiting the phosphatase activity (Sen, Nagy-Zsvér-Vadas et al. 2012). This function is even more obvious in the *Drosophila* epidermis with weaken AJs, shedding light on the redundant function between PATJ and Cadherin complex in modeling cytoskeleton architecture (Sen, Nagy-Zsvér-Vadas et al. 2012).

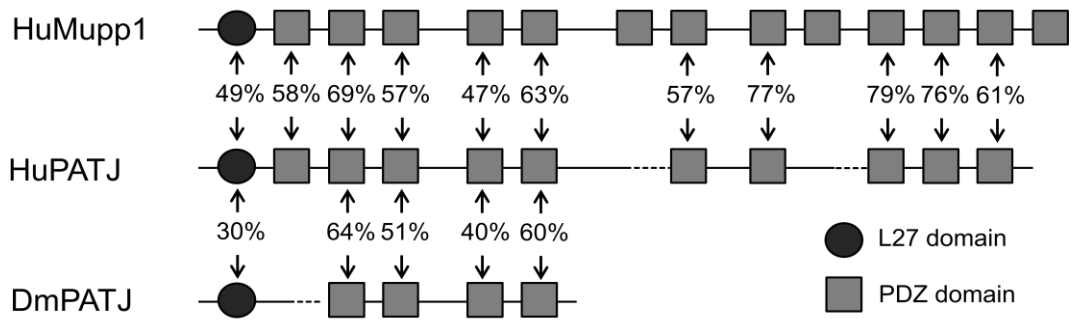

Figure 1.5 Scheme of the structural conservation between PATJ and Mupp1. PATJ and Mupp1 are highly conserved in the protein domains and sharing several common binding partners (adapted from Roh, Makarova et al. 2002).

Two homologous of *Drosophila* PATJ are encoded in mammal systems: PATJ (also named INADL in mouse) and multiple PDZ protein 1 (Mpdz or Mupp1) (Hamazaki, Itoh et al. 2002; Adachi, Hamazaki et al. 2009). Mammalian PATJ has ten PDZ domains and Mupp1 is a bit larger in size, exhibiting thirteen PDZ domains. Although PATJ and Mupp1 share several common binding partners such as JAM1, ZO-3, Pals1, PAR6 and nectins and have similar TJ localization, their functions seem to be differentiated: In mammalian epithelial cells (at least in the analyzed MDCK cells), PATJ is indispensable for the TJ establishment and cell polarization, while the function of Mupp1 seems to be rather mild (Shin, Straight et al. 2005; Adachi, Hamazaki et al. 2009). Interestingly, knock-down of PATJ impairs the *in vitro* cyst formation and attenuates the migrating ability of MDCK cells (Shin, Straight et al. 2005; Shin, Wang et al. 2007). Moreover, PATJ is shown to be important for the proper cytokinesis in a mammary cell line, presumably through regulating (inhibiting) the phosphorylation of myosin regulatory light chain (MRLC) (Bui, Lee et al. 2016).

Lin7 exhibits a similar structure as PATJ, despite the number of PDZ domains. Surprisingly, *lin7* mutant flies are viable and fertile without any defects regarding epithelial polarity, except light-dependent degeneration of photoreceptor cells (Bachmann, Grawe et al. 2008). In contrast, mammalian Lin7 appears to stabilize Pals1 and further ensure TJ formation in MDCK cells (Straight, Pieczynski et al. 2006).

1.3.2 Baz/PAR-3-aPKC complex

Another apical protein complex is the Baz/PAR-3-aPKC complex, consisting of two PAR (partitioning defective) proteins (PAR-3/Baz and PAR-6), atypical PKC (aPKC) and a small GTPase Cdc42. Baz, a homolog of mammalian PAR-3, was the first PAR protein characterized in *Drosophila* and was shown to be important in regulating epithelial polarity as well as the asymmetric division of neural stem cells (Kuchinke, Grawe et al. 1998; Wodarz, Ramrath et al. 1999; Wodarz, Ramrath et al. 2000). Baz acts together with PAR-6/aPKC in the establishment and maintenance of epithelial polarity through forming trimeric complex (Lin, Edwards et al. 2000; Tepass 2012). PAR-6 binds to aPKC through the interaction of their PB1 domains, and the semi-CRIB domain of PAR-6 mediates the binding of Cdc42. It is proposed that binding of Cdc42 by PAR-6 is able to activate the kinase activity of aPKC, phosphorylating Baz/PAR-3 and therefore releases Baz/PAR-3 from the complex (Horikoshi, Suzuki et al. 2009; Graybill et al., 2012; Yamanaka et al., 2001).

Although Baz is thought to act as one of the initiating cues for cellularization of *Drosophila* blastoderm epithelium, a recent study shows that it is dispensable for apical-basal polarity in the follicular epithelium of *Drosophila* (Harris and Peifer 2004; Laprise and Tepass 2011; Shahab, Tiwari et al. 2015).

1.4 Dynamic interactions between apical polarity regulators

Apico-basal polarity is a hallmark of epithelial tissues and is regulated by several evolutionary conserved protein complexes. Many components of these protein complexes are highly dynamic and regulated by various modifications along different developmental stages and in distinct tissues or cell types.

1.4.1 Dynamic composition of protein complexes

The founding members of the major apical polarity regulators were identified in the

screening with worms and flies and led to the common concept of two independent complexes, namely as Baz/PAR-3-aPKC complex and Crb complex (Tepass 2012). However, the last two-decade studies indicate that these two protein complexes are highly dynamic in compositions and far more complicated.

One way to increase this complexity is by encoding more than one isoforms of the polarity gene. As the more precise genome annotations are released, it is predicted that nearly all *Drosophila* polarity genes have more than one isoforms (see FlyBase for more information). A recent study demonstrates the importance of different isoforms of Crb by characterizing a helicase gene mutant *obelus* (Vichas, Laurie et al. 2015). Interestingly, they found that in *obelus* mutant, one *crb* RNA is upregulated during development and the *obelus* phenotype can be mimicked by overexpression of a certain *crb* isoform but not by the other two. This study raises the possibility that different Crb complexes are existing in parallel by simply exchanging the Crb isoforms. Besides, Sdt has four isoforms and these isoforms seem to have different functions and different expression patterns as described above (Bulgakova, Rentsch et al. 2010).

In the light of evolution, things can be more complicated. In mammalian systems, apart from the different splicing strategies, some genes are tending to duplicate themselves, resulting in more than one homologue. PAR-6 is a typical example. In *Drosophila* there is only one PAR-6 gene, while in mammals the number becomes four (Gao and Macara 2004). These different PAR-6 genes appear to have distinct functions in polarity regulation at least in MDCK cells. A recent study shows that PAR6G is a major suppressor of tumorigenesis, but not the other homologous (Marques, Englund et al. 2015).

A second way to modify the complex components is achieved by promiscuous interactions. During gastrulation of *Drosophila* embryonic epidermis, Sdt is able to interact with Baz directly, though the PDZ domain of Sdt and the aPKC binding domain of Baz (Krahn, Bückers et al. 2010). This interaction is proposed to be the important mechanism stabilizing Sdt before the onset of Crb expression during early

embryogenesis. PAR-6 seems to be another versatile protein. It can directly interact with the C-terminal PDZ binding motif of Crb via its PDZ domain and with the third PDZ domain of PATJ through its PB1 domain (Nam and Choi 2003; Hutterer, Betschinger et al. 2004; Kempkens, Médina et al. 2006). Moreover, PAR-6 interacts with Pals1/Sdt. This interaction is mediated by the semi-CRIB-PDZ tandem of PAR6 and the evolutionarily conserved regions (ECRs) of Pals1/Sdt by *in vitro* GST pull-down assays (Hurd, Gao et al. 2003; Wang, Hurd et al. 2004). Apart from these, aPKC binds to the intracellular tail of Crb and PATJ (Sotillos, Díaz-Meco et al. 2004). Binding of aPKC to Crb indices the phosphorylation of Crb and is essential for the maturation of the Crb complex (Sotillos, Díaz-Meco et al. 2004). However, most of these findings have been achieved by using overexpression systems in cultured cells and it still remains to be investigated, whether these different complexes are stably formed *in vivo* and whether they contribute to the apical-basal polarization of epithelial cells.

Additionally, along with intensive studies with respect to cell polarity, novel players with diverse functions are identified. The PDZ binding motif of Crb can interact with α-adaptin which is a component of the AP-2 complex (Lin, Currinn et al. 2015). Upon this interaction, the endocytosis of Crb is strengthened and thought to be important for the degradation of Crb and the dynamic regulation of epithelial polarity. As the Sdt competes for the same binding site and crucial for Crb functionality, it is reasonable to propose that Crb stability and function is balanced by the different intracellular binding partners.

The network becomes even more complicated when all the interactions and dynamic compositions are linked with the specific developmental issues as different developmental stages and different cells behave differently: harboring distinct and dynamic polarity regulators.

1.4.2 Protein modifications

Epithelial polarity regulators are frequently modified in various means.

Phosphorylation is one of the important modifications. It has been reported that in *Drosophila*, phosphorylation of Baz by the basolateral localized kinase PAR1 at Ser151 and Ser1085 prevents the binding of aPKC and inhibits the dimerization of Baz and this phosphorylation can be counteracted by the protein phosphatase 2A (PP2A) (Benton and St Johnston 2003; Krahn, Egger-Adam et al. 2009). Another example is that aPKC phosphorylates Baz at the aPKC binding region (S980) and weakens the binding ability of Sdt and Baz, therefore leading to the dissociation of Sdt from Baz and forming the Crb complex (Krahn, Bückers et al. 2010; Morais de Sa et al. 2010; Walther and Pichaud 2010). Phosphorylations by different kinases seem to provide crucial cues mediating the dynamic changes of protein complex components.

Another way to control protein complex stability lays to the protein quality control, specifically the ubiquitylation dependent protein degradation (Bórquez and González-Billault 2011). To date, many E3 ligases, which directly bind the specific substrates and tag them with a degradation mark, are shown to be important in regulating cell polarity. The F-box E3 ligase Slmb restricts and modulates the activity and stability of aPKC-PAR-6 in *Drosophila* epithelial cells as well as in oocytes (Morais-de-Sá, Mukherjee et al. 2014; Skwarek, Windler et al. 2014). Moreover, another E3 ligase PDZRN3 can induce the poly-ubiquitination of Mupp1 and impair the endothelial cell-cell junction stability in the corresponding overexpression mouse model (Sewduth, Kovacic et al. 2017). Apart from the well characterized E3 ligases, the notch pathway regulator Ebi physically binds to the extracellular domain of Crb and results in the ubiquitin-dependent downregulation of Crb in the dorsoventral (DV) boundary of *Drosophila* imaginal discs (Nguyen, Vuong et al. 2016).

Lastly, the ubiquitination-like modification, sumoylation is also involved in polarity regulation. For instance, Nup358 dependent SUMO modification of aPKC controls the kinase activity of aPKC, which might play a role in the epithelial cell polarity (Yadav, Magre et al. 2016).

1.5 Epithelial polarity and signaling pathways

Epithelial polarity is closely connected to many important signaling pathways. Through these cross-talks, epithelial cells are able to react to extrinsic and intrinsic signals and control the homeostasis and remodeling of tissues as well as the epithelial cells themselves. Here, the signaling pathways which are regulated/regulating epithelial polarity are briefly summarized.

1.5.1 Wnt signaling and epithelial polarity

Wnt proteins are secreted short-range signals which control a variety of developmental processes in all metazoans (Kikuchi, Yamamoto et al. 2011; Niehrs 2012; Swarup and Verheyen 2012; Wang, Sinha et al. 2012). Based on studies in *Drosophila*, *Xenopus*, and zebrafish, Wnt signaling can be briefly divided into the canonical pathway which involves β-catenin mediated transcription and the β-catenin-independent noncanonical Wnt pathways composing of Wnt-planar cell polarity (PCP) and Wnt-Ca^{+2} signaling.

The best-known cross-talking mechanisms between Wnt signaling and epithelial polarity are established on studies into PCP (Yang and Mlodzik 2015; Chu and Sokol 2016). PCP signaling regulates the tissue organization through remodeling the cytoskeleton networks. Dishevelled (Dvl), the adaptor protein of Wnt receptor, can active the kinase activity of aPKC by directly binding, therefore playing important roles in axonal differentiation and polarized migration of mammalian cells (Schlessinger, McManus et al. 2007; Zhang, Zhu et al. 2007). Additionally, PATJ can recruit aPKC to the Wnt receptor Frizzled through direct interactions, resulting in enhanced phosphorylation of Frizzled and inhibited PCP in *Drosophila* wing (Djiane, Yogev et al. 2005). This effect can be further balanced by expressing Baz (Djiane, Yogev et al. 2005). Thus, aPKC mediates many connections between PCP and cell polarity.

1.5.2 Notch signaling and epithelial polarity

Notch signaling defines an evolutionarily conserved mechanism that regulates various developmental events, including growth, cell fate determination and many other functions (Artavanis-Tsakonas, Matsuno et al. 1995; Artavanis-Tsakonas, Rand et al. 1999). Notch signaling is activated by the binding of Notch receptor to its transmembrane ligands and this binding leads to the shedding of the intercellular domain of Notch receptor cutted by γ-secretase, which enters the nucleus and leads to gene transcription (Mumm and Kopan 2000).

Studies in *Drosophila* show that Crb genetically interacts with Notch receptor as demonstrated by larger wing size in heterozygous double mutants (Richardson and Pichaud 2010). This function is achieved presumably by direct interaction between the extracellular domains of Crb and Notch, therefore inhibiting Notch receptor binding to its ligands (Nemetschke and Knust 2016). Apart from Crb, aPKC-iota inhibits stem cell self-renew and promotes differentiation by downregulating Notch signaling in mouse neural stem cells (Mah, Soloff et al. 2015). Moreover, loss of PAR-3 cooperates with Notch signaling in tumorigenesis and enhances tumor invasion and following up metastasis in a Notch receptor intracellular domain (NICD) overexpression model (McCaffrey, Montalbano et al. 2012). The basolateral marker, Lgl negatively regulates Notch signaling by inhibiting the endocytosis of Notch receptor, which contributes to tumorigenesis upon loss of Lgl in mammals (Parsons, Portela et al. 2014; Portela, Parsons et al. 2015).

1.5.3 Hippo pathway and epithelial polarity

The Hippo pathway was first discovered in a screening for regulators of organ size in *Drosophila* (Grusche, Richardson et al. 2010). Follow-up studies showed that this pathway is highly conserved and plays a central role in controlling cell proliferation, apoptosis and stemness in response to various extracellular and intracellular signals, such as cell-cell contacts, cell polarity, mechanical and energy status (Yu, Zhao et al. 2015). The Hippo pathway involves a core kinase cascade which ultimately results in the phosphorylation of the transcriptional coactivators Yorkie (Yki, Yap in mammals)

and TAZ (not conserved in flies), promoting their cytosolic retention (Yu, Zhao et al. 2015).

The upstream activating protein complex, Expanded-Merlin-Kibra, binds to Crb and propagates the Hippo signaling (Chen, Gajewski et al. 2010; Ling, Zheng et al. 2010; Robinson, Huang et al. 2010). Thus, the loss of Crb leads to dephosphorylation of YAP/Yki and enhanced proliferation. In addition, in mammals, the tight junction associated protein Angiomotin (AMOT) directly binds YAP and sequesters it in cytosol independent of the YAP phosphorylation status (Chan, Lim et al. 2011; Zhao, Li et al. 2011). Since AMOT also binds to Pals1, it is interesting to investigate how Pals1 regulates Hippo pathway (Varelas, Samavarchi-Tehrani et al. 2010).

Additionally, a cell adhesion molecule Echinoid (Ed) acts as a tumor suppressor by directly binding the core kinases and enhancing their activity and Yki phosphorylation in *Drosophila* (Yue, Tian et al. 2012).

1.5.4 Other pathways related to epithelial polarity

TGF-β signaling is featured by harboring the serine/threonine kinase receptors and controls cell polarity and cancer progression (Ozdamar, Bose et al. 2005). Upon TGF-β stimulation, TGF-β type II receptor can bind to and phosphorylate PAR6. This phosphorylation creates a docking site for an E3 ligase Smurf1, which mediates ubiquitylation and degradation of RhoA. Loss of RhoA causes cell remodeling and epithelial-to-mesenchymal transition (EMT). This mechanism seems to play an important role in the cancer metastasis. A recent study further shed light on the Pals1 mediated cross-talk between TGF-β signaling and Hippo pathway, emphasizing the central role of cell polarity proteins in linking different pathways (Weide, Vollenbröker et al. 2017).

Polarity proteins are also linked to immune signaling. One study using a mouse mammary cell line shows that loss of PAR-3 leads to enhanced NF-κB signaling mediated by boosted aPKC kinase activity and this cascade finally results in the activity of JAK-STAT axis (Guyer and Macara 2015). However, it is an open question

whether this observation has a biological relevance or not especially upon pathogen invasions.

1.6 Aim of the study

Apico-basal polarity is a significant feature of epithelial tissues and it is regulated by several evolutionarily conserved protein complexes. Among them, PAR-aPKC complex and Crb complex are defining the apical domain of the cell membrane. Interestingly, both complexes are highly dynamic in components during *Drosophila* embryonic development. Hence, it is intriguing to understand how these dynamics are mediated and the impacts on the epithelial cell polarity.

Firstly, the apical targeting mechanism of the core component of the Crb complex PATJ was investigated. Through biochemistry analysis and genetic studies, we identified the existence of the Baz-Sdt-PATJ complex, which is important for apically localization and functionality of PATJ in *Drosophila*.

Secondly, we focused on the role of PAR-6 on regulating the stability of Crb complex. PAR-6 has been shown to interact with the Crb complex in previous studies with overexpression systems of cell lines and/or bacterially purified proteins. In our study, we established a new mechanism of PAR-6 in regulating epithelial polarity, which links protein quality control to the maintenance and establishment of cell polarity in *Drosophila*.

Lastly, a structure-function analysis of Crb-adaptor Sdt was performed due to limited *in vivo* information of protein-interaction features of Sdt. Several genetically modified flies harboring domain-deletion variants of Sdt were generated and the detailed phenotypes were analyzed.

Chapter 1. Upstream regulators of PATJ

Localization and Function of Pals1 Associated Tight Junction Protein in Drosophila is Regulated by Two Distinct Apical Complexes

Arnab Sen*, Rui Sun* and Michael P. Krahn

*These authors contributed equally to the work.

This project aims to understand the apical targeting mechanism of the core component of the Crb complex PATJ. In the embryonic epidermis of *Drosophila*, PATJ is not initially recruited to the apical cell-cell contacts by binding to Crb-Sdt as Crb is not expressed in the early embryogenesis, but depending on forming a new complex with Bazooka (Baz). We found that the Baz-Sdt-PATJ complex exists in parallel to the Crb-Sdt-PATJ complex and is important for the apically targeting of PATJ. These two complexes might play a redundant role in myosin activity modulation.

Author contributions:

Rui Sun: mostly the biochemistry studies, partially the genetic studies and manuscript writing

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Localization and Function of Pals1 Associated Tight Junction Protein in *Drosophila* is Regulated by Two Distinct Apical Complexes

The transmembrane protein Crumbs (Crb) and its intracellular adaptor protein Pals1 (Stardust, Sdt in *Drosophila*) play a crucial role in the establishment and maintenance of apical-basal polarity in epithelial cells in various organisms. In contrast, the multiple-PDZ-domain-containing protein PATJ, which has been described to form a complex with Crb/Sdt, is not essential for apical-basal polarity or for the stability of the Crb/Sdt complex in the *Drosophila* epidermis. Here we show that in the embryonic epidermis Sdt is essential for the correct subcellular localization of PATJ in differentiated epithelial cells but not during cellularization. Consistently, the L27-domain of PATJ is crucial for the correct localization and function of the protein. Our data further indicate that the four PDZ domains of PATJ function to a large extent in redundancy regulating the protein's function. Interestingly the PATJ-Sdt heterodimer is not only recruited to the apical cell-cell contacts by binding to Crb but depends on functional Bazooka (Baz). However biochemical experiments show that PATJ associates with both complexes, the Baz-Sdt and the Crb-Sdt complex in the mature epithelium of the embryonic epidermis, suggesting a role of these two complexes for PATJ's function during development of *Drosophila*.

Keywords: Cell polarity, Adherens junctions, Drosophila, Tight junction, Myosin

INTRODUCTION

Apical-basal polarization of epithelia is regulated by conserved complexes determining the apical versus the basolateral domain (Tepass 2012; Roignot, Peng et al. 2013): Apical to the Adherens Junctions (AJ), the membrane-associated PAR (partitioning-defective) - aPKC (atypical protein kinase C)-complex regulates assembly of the Crumbs(Crb)-complex, which assembles more apically in the so-called subapical region (SAR). The activity of these two complexes is counterbalanced by proteins such as Scribble-Lethal(2) giant larvae-Discs large(Dlg), which localize to the basolateral domain. In the past, various studies have demonstrated that both apical complexes are rather dynamic and that their composition might be tissue-dependent and temporally and/or developmentally regulated (Hurd, Gao et al. 2003; Nam and Choi 2003; Penkert, DiVittorio et al. 2004; Sotillos, Diaz-Meco et al. 2004; Wang, Hurd et al. 2004; Kempkens, Medina et al. 2006; Krahn, Buckers et al. 2010).

In *Drosophila*, the multiple PDZ-domain-containing protein PATJ has been described to function in a complex with Crb and Stardust (Sdt, the *Drosophila* homologue of Partner of Lin-7 one, Pals1) to regulate apical-basal polarity in follicle epithelial cells and photoreceptor cells (Tanentzapf, Smith et al. 2000; Nam and Choi 2006; Richard, Grawe et al. 2006).

In mammalian and *Drosophila* epithelial cells, Pals1/Sdt is recruited by the cytoplasmic tail of Crb to the “subapical region” and in turn stabilizes Crb (Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001; Makarova, Roh et al. 2003; Roh, Fan et al. 2003; Straight, Shin et al. 2004). The L27 domain of Pals1 has been shown to heterodimerize with the L27-domain of PATJ, thereby tethering PATJ to tight junctions (TJ) (Roh, Makarova et al. 2002; Li, Karnak et al. 2004; Feng, Long et al. 2005).

Recently, we and others reported that loss of PATJ in *Drosophila* does not affect apical-basal polarity in the embryonic epidermis or in follicle epithelial cells but

rather modulates Myosin activity to support AJ stability (Penalva and Mirouse 2012; Sen, Nagy-Zsver-Vadas et al. 2012; Zhou and Hong 2012). Exclusively in photoreceptor cells and to some extent in the follicular epithelium, PATJ seems to be essential for the correct subcellular localization of the Crb-Sdt complex, either directly by stabilizing this complex or indirectly by regulating photoreceptor morphology/development (Sen, Nagy-Zsver-Vadas et al. 2012; Zhou and Hong 2012).

Two mammalian orthologues of PATJ are expressed in epithelia: mammalian PATJ (mPATJ, encoded by *INADL* in mice) and Multiple PDZ-domain protein 1 (MUPP1). Both proteins are very similar to DmPATJ: In addition to an N-terminal L27 domain, they exhibit several PDZ domains (DmPATJ four, mPATJ ten, MUPP1 thirteen) and localize to the TJ in mammalian epithelial cells (Adachi, Hamazaki et al. 2009). However, Abachi et al. showed that despite its domain similarity, mPATJ but not MUPP1 regulates TJ stability (Adachi, Hamazaki et al. 2009). These data are in line with previous findings describing TJ-formation delay or defects upon loss of mPATJ in cultured epithelial cells (Michel, Arsanto et al. 2005; Shin, Straight et al. 2005). Other studies describe a role of mPATJ in Myosin-driven processes like apical constriction and cell migration (Shin, Wang et al. 2007; Ernkvist, Luna Persson et al. 2009; Nakajima and Tanoue 2011).

In this study, we report that in the embryonic epidermis of *Drosophila* PATJ assembles with the Crb-Sdt complex but additionally associates with the Baz-Sdt-complex we described previously (Krahn, Buckers et al. 2010). Notably, deletion of Baz and Sdt but not of Crb leads to mislocalization of PATJ during gastrulation and in fully differentiated epithelia of the embryonic epidermis. In contrast, localization of PATJ at the basal junction next to the tip of the invaginating plasma membrane during cellularization is independent of Baz/Sdt. Consequently, deletion of the L27-domain of PATJ leads to an abolished apical accumulation and impaired function of the protein. Studies with chimeric proteins further suggest that targeting of PATJ's PDZ-domains to the Baz-(Sdt) and Crb-(Sdt) complex are sufficient for PATJ's function. Finally, we investigated the functionality of PATJ's four

PDZ domains and provide evidence that under close to endogenous expression levels, none of these domains is essential.

EXPERIMENTAL PROCEDURES

Drosophila genetics - The following mutant alleles were used: *PATJ*$^{\Delta 1}$ (Sen, Nagy-Zsver-Vadas et al. 2012), *baz*$^{815-8}$ (McKim, Dahmus et al. 1996; Krahn, Klopfenstein et al. 2010), *baz*^{XR11} (Shahab, Tiwari et al. 2015), *sdt*^{K85} (Berger, Bulgakova et al. 2007) and *crb*^{11A22} (Tepass and Knust 1990). Germ line clones were generated with the mutant alleles recombined with FRT using the dominant female sterile technique (Chou, Noll et al. 1993). Homozygous mutant embryos were identified using antibody stainings. Follicle cell clones were generated with the FRT-Flp system as described before (Sen, Nagy-Zsver-Vadas et al. 2012). Ubi::PATJ-GFP (mutant/chimeric) constructs were generated using phiC31-mediated germ line transformation using attP40.

DNA and constructs - The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate domain deletions with full-length PATJ cDNA in pENTR (Sen, Nagy-Zsver-Vadas et al. 2012) as a template. The following oligonucleotides were used for mutagenesis:

PATJΔL27: 5'- GCGGATATTTCCAGCTCCAT-GTTGCCCAAC-3'; PATJΔPDZ1: 5'-GCCATA-GAGCTGGTCCGTCCCGTTGAGCAG-3'; PATJΔPDZ2: 5'-GAAACGGAGAAGCTTCGC-TACCTGAGGGGC-3'; PATJΔPDZ3: 5'-GGCT-CCGATGTGGAGTGCGGTCGCAACAGG-3';

PATJΔPDZ4: 5'-ATGTGGTCGTCCCAACGC-ATTGGTGTGGCC-3';

To generate truncated versions of PATJ, the following primers were used: PATJ-F: 5'-CACCATGCACCTCAGCGCGGA-3'; PATJ-151-R: 5'-CTCTATGGCCTGGATCTGAGC-3';

PATJ-256-R: 5'-CAGGGCGTACTGGGG-3';

PATJ-449-R: 5'-TGATGGTGTAGTTGTGGC-3'

For $PATJ_{\Delta L27\ PDZ(Sdt)}$, the PDZ domain of Sdt was amplified with Sdt-PDZ-F: 5'-GCGGCCGCCCCCTTCACCATGCGTATCATCCAGATCGAG-3' and Sdt-PDZ-R: 5'-GCGGCCGCCGGTGGACTACCCGCTGG and inserted with NotI (underlined) into $PATJ_{\Delta L27}$ pEntry. Similarly, the PDZ domain of PAR6, the oligomerization domain of Baz and the FERM-domain of Yurt were cloned into NotI of $PATJ_{\Delta L27}$ pEntry using the following oligonucleotides: PAR6-PDZ-F: 5'-GCGGCCGCCCCCTTCACCATGAGAAGAGTGCGGCTACTG-3', PAR6-PDZ-R: 5'-GCGGCCGCCTTCACGGTGATTATCAGATTG-3', Yrt-FERM-F: 5'-GCGGCCGCCCCCTTCACCATGGTGCTCGGAAAGGATGGC-3', Yrt-FERM-R: 5'-GCGGCCGCTTTGACCGGCGCCC TAA-3'; Baz-CR1-F: 5'-GCGGCCGCCCCCTTCACCATGAAGGTCACCGTCTGCTTCGGC-3', Baz-CR1-R: 5'- GCGGCCGCATCTCCGCC TCCTTGC-3'. $Baz_{733-1221}$ was cloned into an endogenous SacII site (aa 633) of $PATJ_{\Delta L27}$. All constructs were recloned into destination vectors (modified UWG, Murphy lab, DGRC) using the gateway technology (Life technologies).

Immunoprecipitation and Western blotting - For immunoprecipitations, w^- embryos from an overnight collection were dechorionated and lysed in lysis buffer (1% Triton X-100, 150mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 50mM TRIS-HCl pH 7.5) supplemented with protease inhibitors. After centrifugation, 2 µl of rabbit anti-Baz (Wodarz, Ramrath et al. 1999) or 2 µl of the corresponding preimmune serum were added to the embryonic lysate, corresponding to 500 µg total protein. Immune complexes were harvested using protein A-conjugated agarose (BioVision). GFP-binder (Chromotek) was used to immunoprecipitate Crb-GFP. Wild-type flies served as control. Beads were washed five times with lysis buffer and boiled in 2x SDS sample buffer before SDS-PAGE and Western blot. Western blotting was done according to standard procedures. Primary antibodies used for Western blotting were as follows: mouse anti Crb (Cq4, 1:50, developed by E. Knust (Tepass, Theres et al. 1990) was obtained from the Developmental Studies Hybridoma Bank (DSHB),

created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242), guinea pig anti PATJ (1:1000, Sen, Nagy-Zsver-Vadas et al. 2012), mouse anti Sdt (1:20, Berger, Bulgakova et al. 2007), rabbit anti-Baz (1:2000, Wodarz, Ramrath et al. 1999) and mouse anti-GFP (B2, 1:500, Santa Cruz, sc-9996).

Immunohistochemistry - Embryos were fixed in 4% formaldehyde, phosphate buffer pH 7.4 as described previously (Krahn, Egger-Adam et al. 2009). Primary antibodies used for indirect immunofluorescence were as follows: rabbit anti-Baz (1:1000, Wodarz, Ramrath et al. 1999), mouse anti Crb (Cq4, 1:50, DSHB), mouse Dlg (1:50, DSHB (Parnas, Haghighi et al. 2001)), guinea pig anti PATJ (1:500, Sen, Nagy-Zsver-Vadas et al. 2012), mouse anti Sdt (1:20, Berger, Bulgakova et al. 2007), guinea pig anti PAR6 (Kim, Gailite et al. 2009), guinea pig anti Yrt (Laprise, Beronja et al. 2006), rabbit anti-GFP (#A11122, 1:1000, Life technologies), rabbit anti-GFP (sc-8334, Santa Cruz Biotechnology) and chicken anti-GFP (1:2000, Aves Laboratories). Secondary antibodies conjugated with Alexa 488, Alexa 568 and Alexa 647 (Life technologies) were used at 1:400.

Images were taken on a Zeiss LSM 710 Meta confocal microscope and processed using Adobe Photoshop.

RESULTS AND DISCUSSION

PATJ is recruited by Sdt to a complex with Baz at the apical junctions in the embryonic epidermis - Upon the formation of apical AJ in late cellularization/early gastrulation in *Drosophila*, PATJ is recruited to the apical cell-cell contact region whereas staining at the basal membrane ceases (Sen, Nagy-Zsver-Vadas et al. 2012). Studies in *Drosophila* and cultured mammalian epithelial cells showed that PATJ associates with Sdt/Pals1 which in turn binds to the transmembrane protein Crb, which targets the complex to TJ in vertebrates and in the corresponding “subapical region” (SAR) in *Drosophila* (Klebes and Knust 2000; Roh, Makarova et al. 2002).

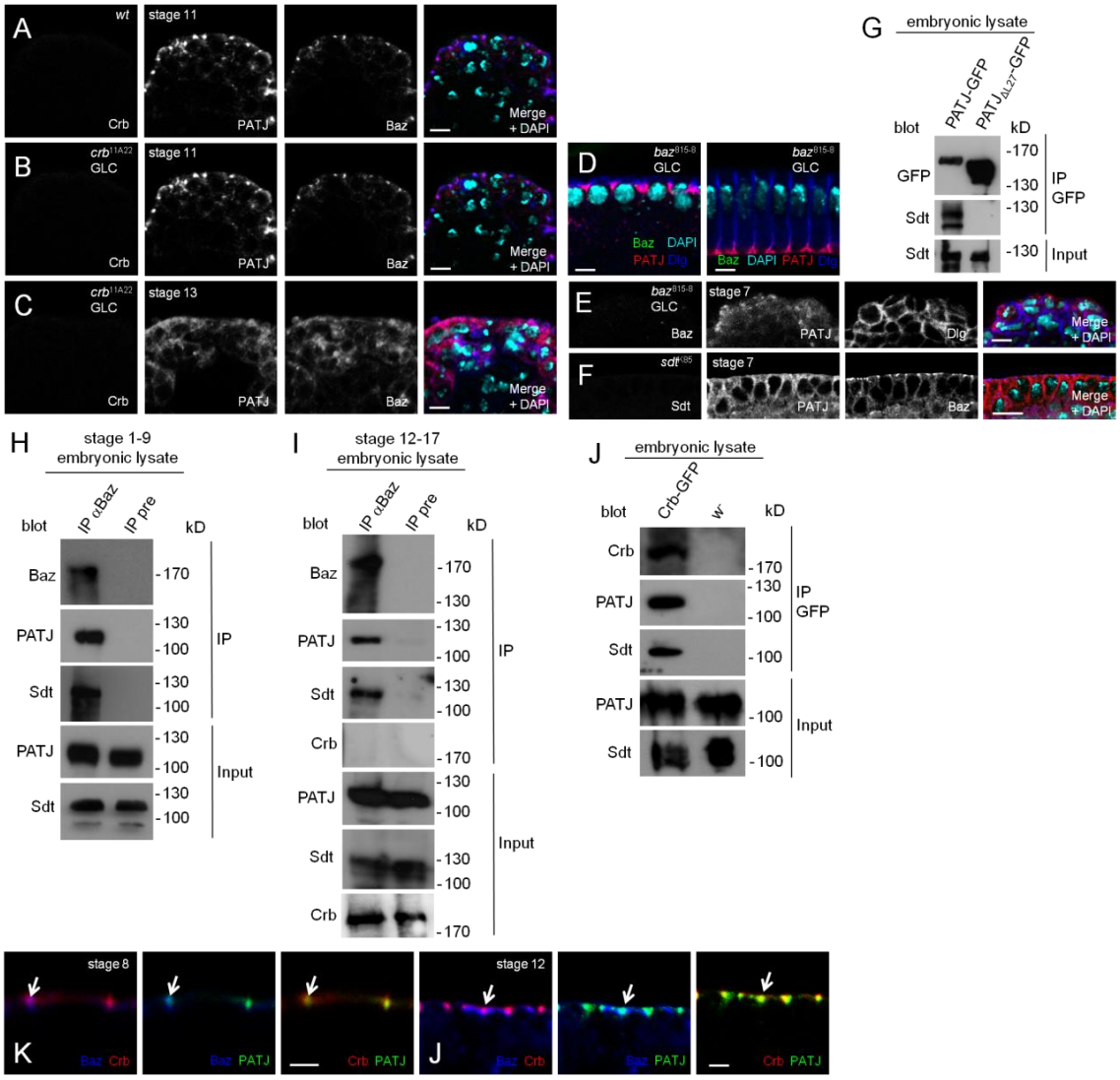


Figure 2.1 A. Endogenous PATJ colocalizes with Crb and Baz at the apical junctions in epithelial cells of the embryonic epidermis. Green: Crb, red: PATJ, blue: Baz. B. Endogenous PATJ localizes at the apical junctions in the absence of Crb expression during early embryogenesis. Green: Crb, red: PATJ, blue: Baz. C. Upon disruption of epithelial integrity in crb-mutant embryos (maternally and zygotically mutant, derived from GLCs) of later stages, Baz as well as PATJ are mislocalized to the cytoplasm. Green: Crb, red: PATJ, blue: Baz. D. PATJ localization during cellularization is not affected in baz-mutant embryos derived from baz815-8 germ line clones. Green: Baz, red: PATJ, blue: Dlg. E. Loss of maternal and zygotic Baz in gastrulation (baz815-8 germ line clones) results in a disturbed apical-basal polarity and cytoplasmic PATJ localization. Green: Baz, red: PATJ, blue: Dlg. F. PATJ is not recruited to the apical junctions in the absence of Sdt. Green: Sdt, red: PATJ, blue: Baz. G. Endogenous Sdt co-immunoprecipitates with PATJ-GFP but not with PATJ□L27-GFP. H and I. Endogenous PATJ co-immunoprecipitates with Baz in early stages (stage 1-9, H) and late stages (stage 12-17, I). Preimmune serum (pre) served as negative control. J.

Co-immunoprecipitation of PATJ with Crb-GFP expressed from its endogenous promoter from embryonic lysates. Wild-type flies served as negative control. K and L. High magnifications of stainings of wild-type embryos with PATJ, Crb and Baz reveal a segregation of Baz and Crb, which starts in earlier embryonic stages (stage 8, K) and gets more pronounced in later stages (stage 12, L). Remarkably, PATJ overlaps with both proteins in both stages (arrows). Scale bars = 5µm in A-F, 2µm in K and L.

We recently found that in the embryonic epidermis of *Drosophila*, Sdt is initially localized to the apical junctions in early gastrulation before Crb is expressed and even remains at the junctional region of epithelial cells when Crb is absent (Krahn, Buckers et al. 2010). This is accomplished by a direct interaction of the PDZ-domain of Sdt with Baz. Upon phosphorylation of Baz by aPKC at Serine 980, Sdt is released from Baz and becomes available to stabilize the Crb complex (Krahn, Buckers et al. 2010). We, therefore, tested whether the subcellular localization of PATJ is dependent on Crb or Baz or both. In maternal and zygotic *crb*-mutant embryos (crb^{11A22} GLC), PATJ shows a correct localization not only during cellularization (data not shown) but also after gastrulation as long as apical-basal polarity is still intact (stage 6-11, Fig. 2.1B, compared to wild-type embryos in Fig. 2.1A). In later stages (from stage 11/12 on), the apical-basal polarity is impaired due to the loss of Crb, finally resulting in a multilayered epithelium. In these cells, PATJ staining is cytoplasmic or in aggregates (Fig. 2.1C). Notably, the loss of cortical PATJ in these embryos is accompanied by a loss of membrane-associated Baz (Fig. 2.1C).

In contrast, in maternal and zygotic *baz*-mutant embryos (baz^{815-8} GLC), the accumulation of PATJ at the basal junction next to the tip of the furrow canal during plasma membrane invagination is not affected (Fig 2.1D) but the targeting of the protein to the apical junctional region after cellularization is abolished (Fig. 2.1E).

Furthermore, we found endogenous PATJ and Sdt to coimmunoprecipitate with endogenous Baz from embryonic lysates (Fig. 2.1H and I). Consequently, in embryos lacking Sdt, PATJ is correctly localized during cellularization (data not shown) but fails to relocalize to the apical junctions during gastrulation (Fig. 2.1F), indicating that PATJ is recruited by Sdt to the apical junctions. Furthermore, deletion of the L27

domain of PATJ results in a disturbed association with endogenous Sdt (Fig. 2.1G). This is consistent with studies in cultured mammalian cells demonstrating that PATJ directly binds to Pals1 via heterodimerization or hetero-oligomerization of its L27 domain with the (more N-terminal) L27 domain of Pals1 (Roh, Makarova et al. 2002; Li, Karnak et al. 2004; Feng, Long et al. 2005). Besides its association with Baz-Sdt, PATJ can also be co-immunoprecipitated with Crb-GFP expressed from its endogenous promoter (Klose, Flores-Benitez et al. 2013, Fig. 2.1J), pointing to the existence of a second complex consisting of Crb-Sdt-PATJ. However, Crb cannot be found to co-immunoprecipitate with Baz (Fig. 2.1I), demonstrating that there is no quaternary complex Baz-Crb-Sdt-PATJ.

To test whether both PATJ-containing complexes are formed in a stage-dependent manner, Baz co-immunoprecipitation experiments were performed either with early (stage 1-9) or late (stage 12-17) stage embryos. Interestingly, PATJ and Sdt can be co-immunoprecipitated with Baz from lysates of early and late developmental stages (Fig. 2.1H and I). This suggests that PATJ is initially recruited to the apical junctions by the Baz-Sdt-complex, whereas the Crb-Sdt-PATJ complex is formed later in development as soon as Sdt is released from Baz upon phosphorylation by aPKC (Krahn, Buckers et al. 2010). However, in late developmental stages, PATJ and Sdt can still be co-immunoprecipitated with Baz. This observation and the facts that PATJ still associates with Baz and remains correctly localized in the absence of Crb in later stages (stage 10/11, Fig. 2.1B) indicate that a portion of Baz remains unphosphorylated by aPKC and associates with Sdt-PATJ. Thus apical localized Baz can complement Crb's function regarding the targeting of Sdt/PATJ throughout embryogenesis. Indeed, high magnification of Baz-Crb-PATJ stainings in late-stage embryos (stage 12) show a partial overlap of PATJ with both proteins (arrows), whereas Baz and Crb are more clearly separated (Fig. 2.1K). In earlier stages of epithelial development (stage 8), the segregation of Baz/Crb is less pronounced and PATJ colocalizes with both proteins (Fig. 2.1K). These data are in line with previous studies, describing the segregation of AJ-associated Baz from Crb, which localizes

more apical to the SAR (Harris and Peifer 2005; Morais-de-Sa, Mirouse et al. 2010).

PATJ localization in the follicular epithelium depends on Sdt and on Crb but not on Baz - Similar to the embryonic epidermis loss of Sdt in the epithelial cells surrounding the oocyte (follicular epithelium) abolishes apical accumulation of PATJ (Fig 2.2A, mutant clones are marked by the absence of RFP). In contrast in *baz*-mutant clones, Sdt, PATJ and Crb are correctly localized to the apical junctions (data not shown and Fig. 2.2B, mutant clones are marked by the absence of Baz staining), which is in line with recent results showing that *baz* null alleles do not exhibit polarity defects (Shahab, Tiwari et al. 2015).

In *crb*-defective follicle cells, apical Sdt and PATJ staining are drastically reduced (Fig. 2.2C, D, arrows), which is partly in line with previous data (Tanentzapf, Smith et al. 2000). Notably, Baz localization is also affected upon removal of Crb in the follicular epithelium (Fig. 2.2D). Thus, the follicular epithelium represents a phenotypic characteristic which differs from the epidermis (PATJ localization is only dependent on Baz-Sdt but not on Crb-Sdt) and resembles rather pupal photoreceptor cells, in which PATJ localization depends on Crb (Pellikka, Tanentzapf et al. 2002; Richard, Grawe et al. 2006). Notably, in photoreceptor cells, PATJ seems to be crucial for the stabilization of the Crb-Sdt complex (Nam and Choi 2006; Richard, Grawe et al. 2006; Zhou and Hong 2012), whereas this phenotype is much weaker in the follicular epithelium: In follicle epithelial cells, loss of PATJ results in decreased apical-junctional accumulation of Crb/Sdt but without subsequent disassembling of the complex and polarity defects. In the embryonic epidermis, loss of PATJ does not affect Crb/Sdt localization or apical-basal polarity (Penalva and Mirouse 2012; Sen, Nagy-Zsver-Vadas et al. 2012; Zhou and Hong 2012).

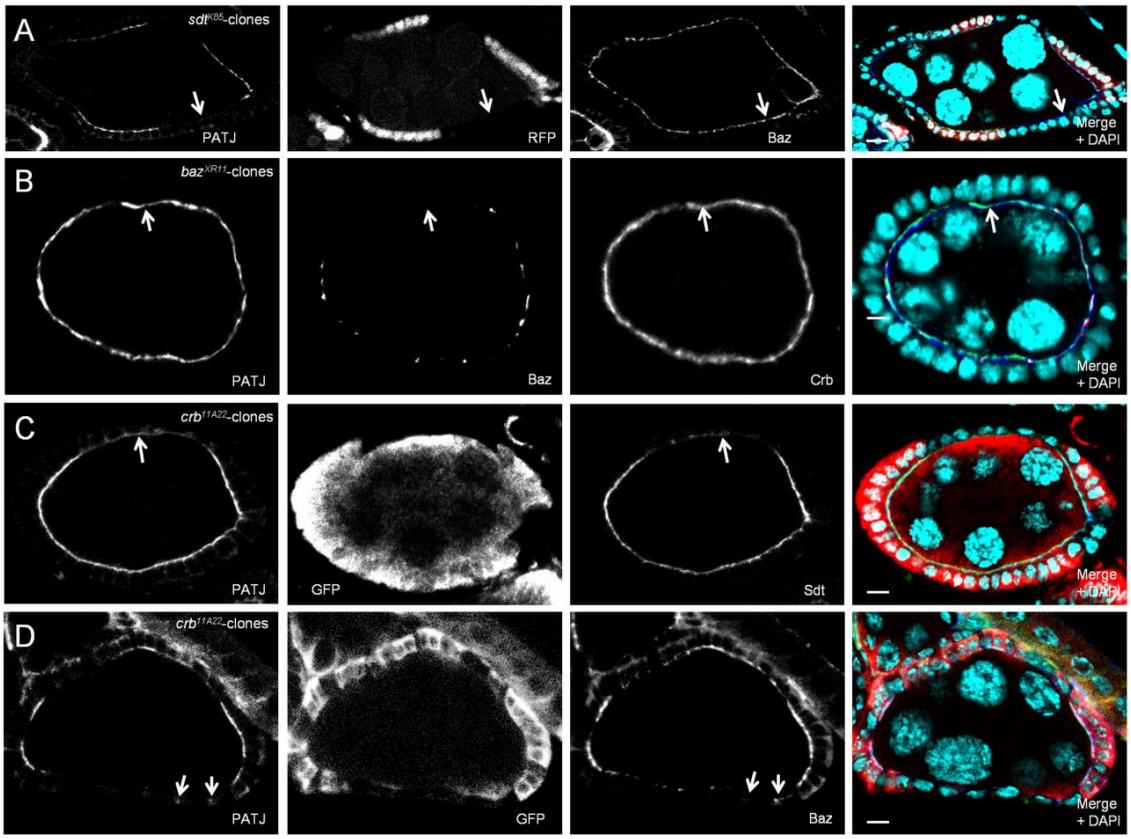


Figure 2.2 PATJ is lost from the apical junctions in sdt- (A) but not in baz- (B) mutant clones (arrows). PATJ localization is only partly retained in crb-mutant clones (C, arrows). D. In crb-mutant follicle cells, localization of Baz to the apical junctions is diminished (arrows). Mutant cells were generated using the FRT-Flp technique and marked by the absence of RFP (A), Baz (B) or GFP (C and D), respectively. Note flat cells in sdt- and crb-mutant cells (A, C and D). Anterior is left in all egg chambers. Scale bars = 10µm.

The L27 domain is essential and sufficient for apical junctional localization - To test which domains are crucial for PATJ's correct subcellular localization and function, we generated deletion constructs of the N-terminal L27 domain and each of the PDZ domains as well as truncated versions of PATJ, all C-terminally tagged with GFP (Fig. 2.3A). To avoid artificially increased protein levels, we expressed the modified proteins under a ubiquitous promoter (Ubiquitin) and used the PhiC31-Integrase system (Groth, Fish et al. 2004) to generate transgenic lines with the identical genomic background, ensuring comparable protein levels (Fig. 2.3B). Indeed, wild-type PATJ-GFP expressed by this system shows similar levels as endogenous

PATJ (Fig. 2.3B). The exogenous protein is localized indistinguishably from endogenous PATJ (Fig. 2.3C) and it is capable of rescuing the *PATJ*$^{\Delta 1}$ null allele to a large extent (79% surviving flies, Fig. 2.3A).

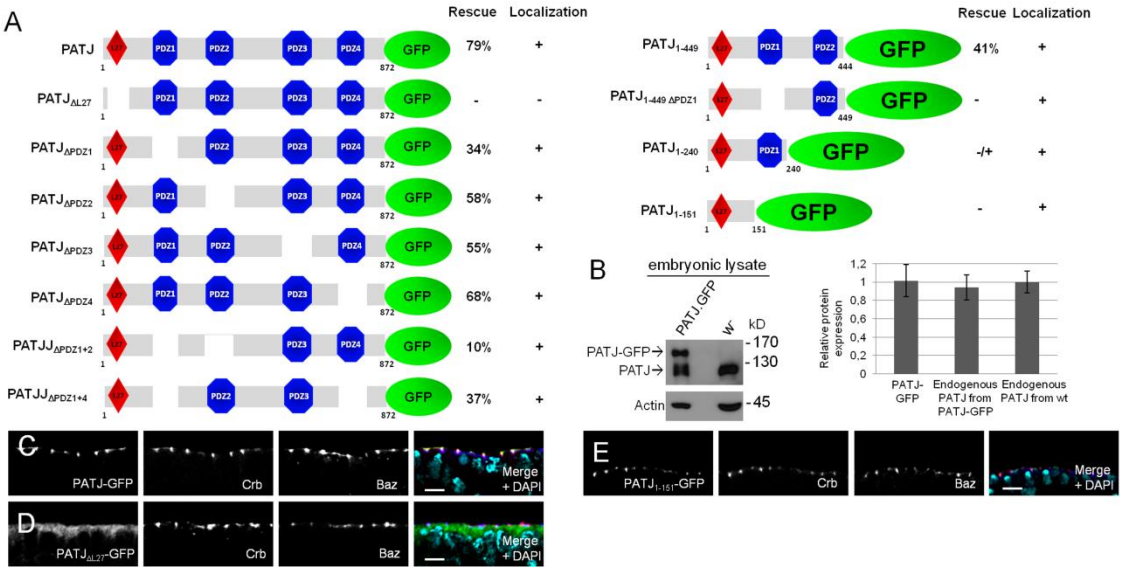


Figure 2.3 A. Schematic drawing of different PATJ constructs tested in this study. The capacity to correctly localize to the apical junctions and to rescue a PATJ null allele (maternal and zygotic mutant *PATJ*$^{\Delta 1}$, n = 300) is indicated. B. Western blot on embryonic lysates from Ubi::PATJ-GFP versus wild-type flies with anti-PATJ antibody indicates that PATJ-GFP is expressed at similar levels as the endogenous protein. An equal amount of total protein was loaded as verified with the anti-actin western blot. Results of three independent experiments were quantified using a chemiluminescence scanner and depicted in B as mean ± SE. PATJ-GFP (C) localizes to the apical junctions indistinguishable from endogenous PATJ, whereas the deletion of the L27 domain (D) disrupts junctional accumulation. E. The isolated L27 domain is sufficient to localize to a far extent at the apical junctions. In all panels, GFP is depicted in green, Crb in red and Baz in blue. Scale bars = 5µm.

In mammalian epithelial cells, mPATJ has been shown to be targeted by Pals1 to the TJ via a heterodimerization of its L27 domains (Roh, Makarova et al. 2002; Li, Karnak et al. 2004; Straight, Shin et al. 2004). Likewise, deletion of the L27 domain of *Drosophila* PATJ results in an abolished association with Sdt (Fig. 2.1G) and a cytoplasmic accumulation of the mutant protein in the embryonic epidermis as well as in follicle cells (Fig. 2.3D and data not shown). Consequently, the PATJ$_{\Delta L27}$-GFP is

unable to rescue the *PATJ*-null allele, resulting in similar phenotypes as the null allele (*PATJ*$^{\Delta 1}$, pupal lethality).

In contrast to deletion of the L27-domain, the removal of any of the four PDZ domains alone does not impair the subcellular localization of the modified protein at the apical junctions (Fig. 2.4A-D). Furthermore, ubiquitous expression of all single deletion constructs can complement PATJ's function and can be maintained as a stable stock with the homozygous *PATJ*$^{\Delta 1}$ allele. However, quantification of the rescue capacity showed that the deletion of the first PDZ-domain affects the functionality of the protein far more than deletion of PDZ2, 3 or 4 (34% in comparison to 58, 55, 68%, respectively, Fig. 2.3A).

As overexpression of a truncated version of PATJ has been reported to be capable of rescuing a PATJ-mutant to some extent (Nam and Choi 2006; Richard, Grawe et al. 2006; Penalva and Mirouse 2012), we determined which minimal region of PATJ is sufficient for the protein's function: As expected, ubiquitous expression of the isolated L27 domain ($PATJ_{1\text{-}151}$) shows a mostly apical localization (Fig. 2.3E). This protein, lacking all PDZ domains, does not rescue the PATJ null allele. Experiments with flies lacking zygotic PATJ expression and ubiquitously expressed $PATJ_{1\text{-}240}$-GFP (L27 domain and the first PDZ domain, Fig. 2.4H) occasionally produced adult flies. The majority of homozygous flies died during late pupal stages but in contrast to the null allele, pupae in the $PATJ_{1\text{-}240}$ rescue underwent complete morphogenesis and died only shortly before hatching (or failed to hatch). Hatched flies were sterile and died after a few days, indicating that the truncated version exhibits sufficient functionality to overcome the pupal lethality of *PATJ*$^{\Delta 1}$ but it is not capable of fully replacing the wild-type protein. Overexpression of the same construct using arm::GAL4 resulted in increased rescue capacity, and the rescued flies can be maintained as a stable stock. Thus only artificially increased levels of the protein consisting of the L27 domain and the first PDZ domain can accomplish the function of PATJ during development, which is in line with previous studies using overexpressed proteins (Nam and Choi 2006; Penalva and Mirouse 2012).

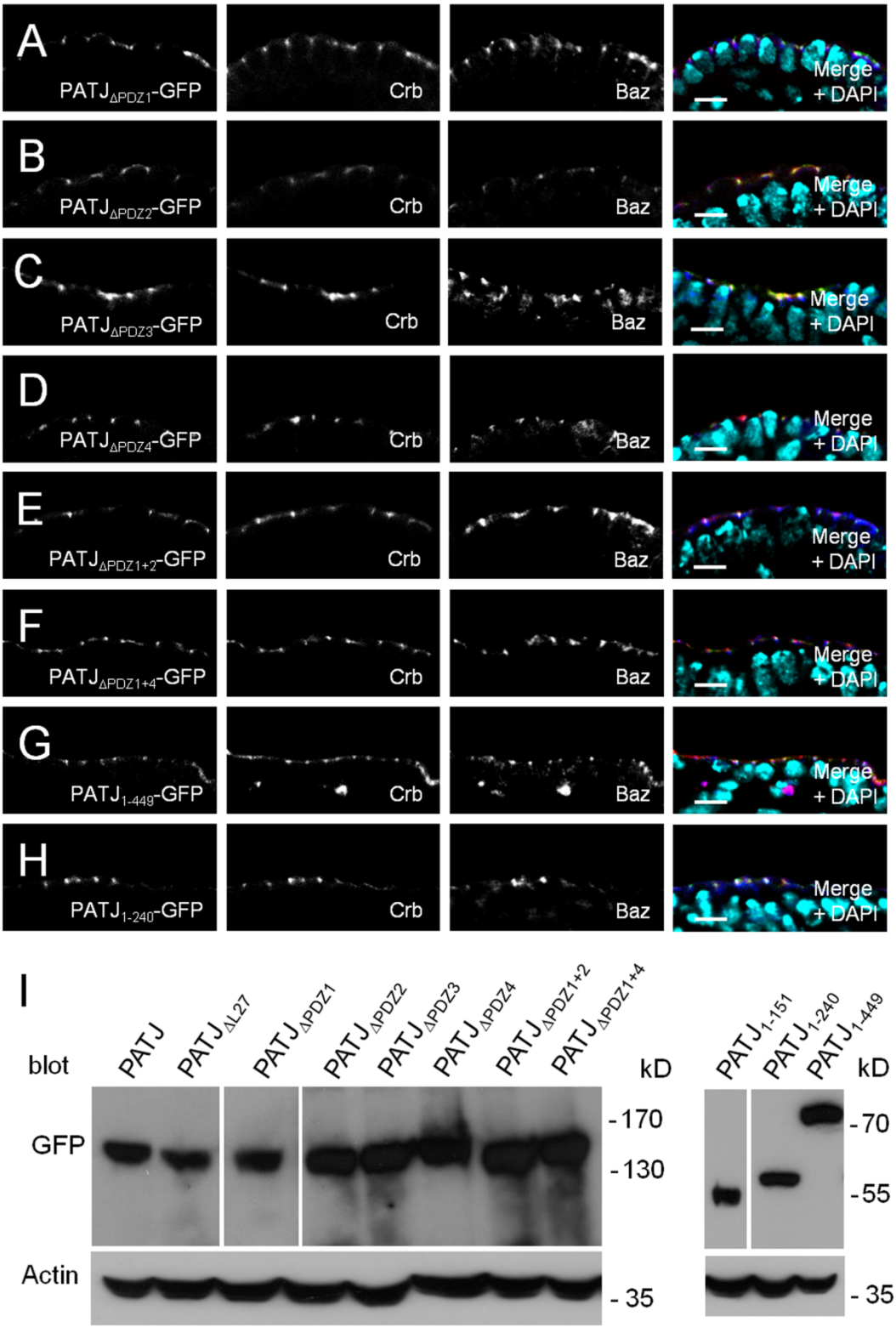


Figure 2.4 A-H. PATJ deletion and truncation proteins expressed with a ubiquitous promoter were stained in the embryonic epidermis using a GFP-antibody (green). Co-immunostainings with endogenous Crb (red) and Baz (blue) reveal a partly overlap of these proteins. I. Expression of PATJ deletion and truncation proteins was tested in Western-Blotting using a GFP-antibody. Scale bars = 5µm.

In contrast to $PATJ_{1-240}$, a protein consisting of the first 449aa, including the L27 domain and the first two PDZ domains ($PATJ_{1-449}$, Fig. 2.4G), expressed at close-to-endogenous levels, can fully rescue the PATJ null allele (Fig. 2.3A). Rescued flies can be kept as a stable stock. Deletion of the first PDZ domain in this construct (resulting in $PATJ_{1-449\ \Delta PDZ1}$-GFP) results again in a loss of functionality as seen in rescue experiments.

These results suggest that none of the PDZ domains is essential for the viability of the fly. Under overexpression conditions, the first PDZ-domain is sufficient for the viability of the fly. This is further supported by the observation that upon deletion of the first two PDZ domains ($PATJ_{\Delta PDZ1+2}$, Fig. 2.4E) or the first and the fourth PDZ domain ($PATJ_{\Delta PDZ1+4}$, Fig. 2.4F), the mutated protein can still rescue $PATJ^{\Delta 1}$. However, survival rates (Fig. 2.3A) indicate that deletion of more than one PDZ domain strongly reduces PATJ's functionality.

Taken together, our data revealed that none of the four PDZ domains is essential for the survival of the fly, although they seem to play a more subtle role during *Drosophila* development as suggested by the different rescue capacities. Taking into account that all four PDZ-domains exhibit only 50-60% sequence similarity (sum of identical and similar amino acids), these results are remarkable.

Association with both Crb-Sdt and Baz-Sdt complexes rather than apical junctional localization is essential for PATJ's function - In order to test whether the association of PATJ with junctional Baz/Crb is crucial for its function or whether an apical junctional accumulation is sufficient, we cloned the PDZ-domain of Sdt to $PATJ_{\Delta L27}$-GFP ($PATJ_{\Delta L27\text{-}PDZ(Sdt)}$, Fig. 2.5A). This domain has been reported to bind to Crb and to Baz (Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001; Krahn, Buckers et al. 2010).

We verified the interaction with both proteins in transgenic flies (Fig. 2.5I). Notably, the localization of this chimeric protein is more or less cytosolic with only a minor

fraction accumulating at the apical junctions (Fig. 2.5B). This might be due to the fact that the protein level of Sdt is restrictively controlled: Even a moderately increased protein amount leads to an entirely cytosolic localization of Sdt (data not shown).

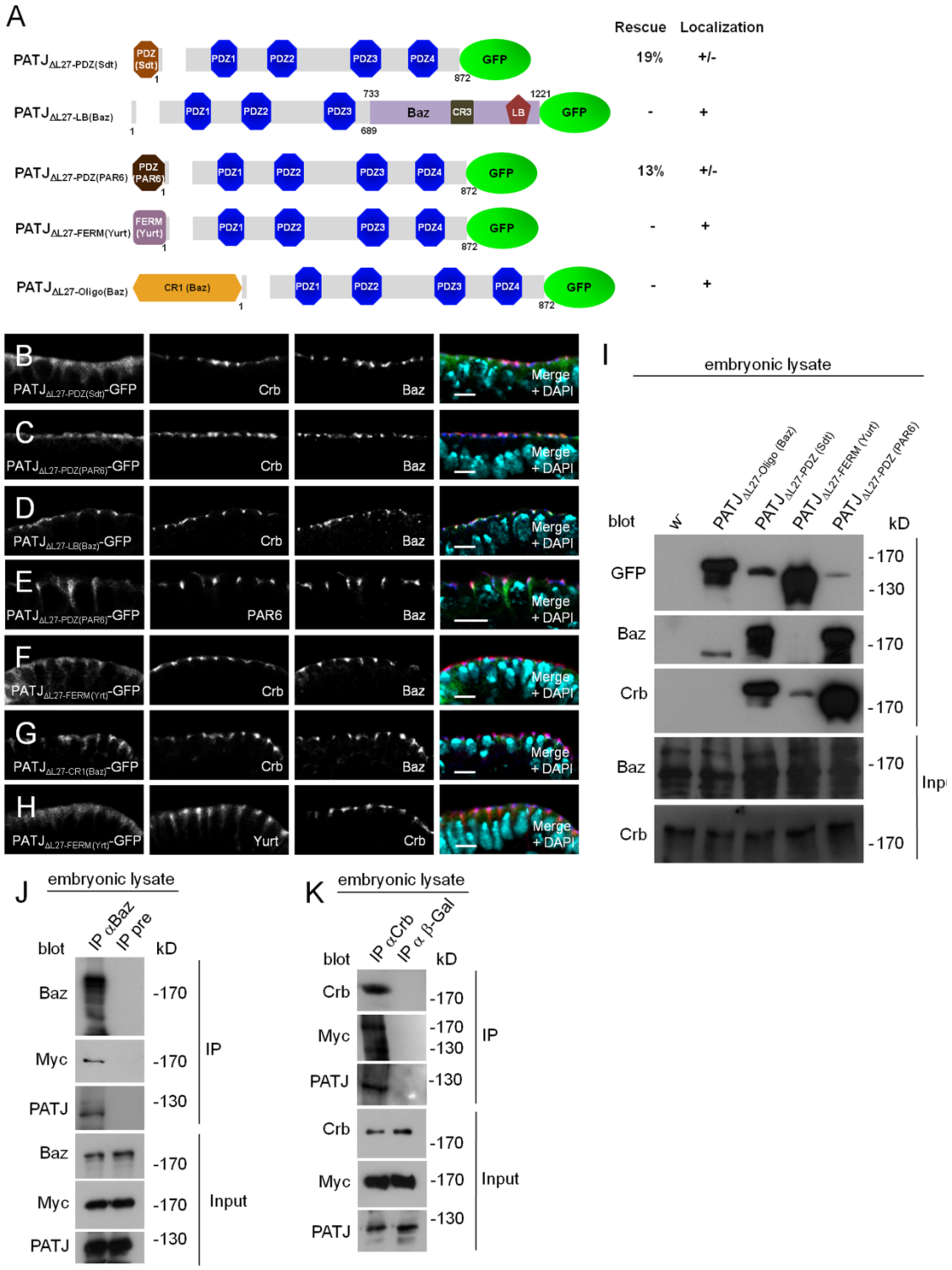


Figure 2.5 A. Schematic drawing of different PATJ constructs tested in this study. The capacity to correctly localize to the apical junctions and to rescue a PATJ null allele

(maternal and zygotic mutant *PATJ*$^{\Delta 1}$, n = 300) is indicated. B-D, F and G. Subcellular localization of chimeric proteins described in A reveals only partly colocalization of the chimeric proteins with endogenous Crb and Baz. GFP is depicted in green, Crb in red and Baz in blue. Scale bars = 5µm. E and H. Immunostaining with a PAR6 or Yrt antibody (both in red) demonstrates a normal localization of these proteins in embryos lacking endogenous PATJ and expressing $PATJ_{\Delta L27\text{-}PDZ(PAR6)}$ or $PATJ_{\Delta L27\text{-}FREM(Yrt)}$, respectively. Chimeric PATJ protein is depicted in green and Baz in blue. Scale bar = 5µm. I. Embryonic lysates (500µg total protein) from PATJ-chimeric protein expressing flies were subjected to immunoprecipitation using a GFP antibody followed by western blotting with the indicated antibodies. Wild-type flies served as negative control. Notably, although all constructs were expressed with the same promoter (Ubiquitin) from the same landing site (attP40) and the same amount of total protein was used for the Western Blot, some chimeric proteins are weaker expressed, suggesting either differences in mRNA stability/degradation or a posttranslational mechanism. J and K. MBS-myc (expressed via the UAS/GAL4 system with da::GAL4) and PATJ co-immunoprecipitate with endogenous Baz (J) and Crb (K).

Nonetheless $PATJ_{\Delta L27\text{-}PDZ(Sdt)}$ restores to some extent the rescue capacity of the protein (19% hatching flies, Fig. 2.5A). The addition of the PDZ domain of PAR6, which is capable of directly binding to both Baz (Joberty, Petersen et al. 2000; Lin, Edwards et al. 2000) and Crb (Lemmers, Michel et al. 2004; Kempkens, Medina et al. 2006) (Fig. 2.5I) to $PATJ_{\Delta L27,}$ results in an apical accumulation of the chimeric protein, although a substantial amount is still cytosolic (Fig. 2.5C). $PATJ_{\Delta L27\text{-}PDZ(PAR6)}$ rescues the PATJ null allele similarly to $PATJ_{\Delta L27\text{-}PDZ(Sdt)}$ (13% hatching flies, Fig. 2.5A). We verified that the low rescue capacity in the strains expressing the described chimeric proteins is not due to mislocalization of endogenous PAR6 (Fig. 2.5E) and Sdt (indirect evidence as Crb is not mislocalized as it would be if Sdt is lost from the SAR, Fig. 2.5B).

In contrast, a protein composed of the three PDZ-domains of PATJ and a fragment of Baz, which accumulates at the apical junctions by direct binding to the plasma membrane (Krahn, Klopfenstein et al. 2010), is to a large extent correctly targeted to the apical junctions ($PATJ_{\Delta L27\text{-}LB(Baz)}$, Fig. 2.5D) but does not rescue the *PATJ*-null allele (Fig. 2.5A). Remarkably, $PATJ_{\Delta L27\text{-}LB(Baz)}$ protein expression is lower in comparison to $PATJ_{\Delta L27\text{-}PDZ(Sdt)}$ and $PATJ_{\Delta L27\text{-}PDZ(PAR6)}$ chimeric proteins. However,

even upon overexpression of the chimeric protein, $PATJ_{\Delta L27\text{-}LB(Baz)}$ cannot rescue the PATJ null allele (data not shown).

As outlined above, Baz is essential to initially recruit Sdt to apical junctions; in later stages, this complex (partly) disassembles by phosphorylation of Baz by aPKC, resulting in apically enriched Sdt which is capable of stabilizing Crb. To dissect whether PATJ exhibits its function through the Baz-Sdt or via the Crb-Sdt complex, we established chimeric PATJ proteins lacking the Sdt-binding domain and exhibiting either a Crb-binding domain (FERM domain of Yurt (Yrt), Laprise, Beronja et al. 2006) or a Baz-binding domain (oligomerization domain CR1, Benton and Johnston 2003; Desai, Sarpal et al. 2013). Both chimeric proteins do not exhibit a dominant negative phenotype by mislocalizing endogenous Baz or Yrt (Fig. 2.5G and H).

Interestingly, although $PATJ_{\Delta L27\text{-}CR1(Baz)}$ and $PATJ_{\Delta L27\text{-}FERM(Yurt)}$ localize at least to some extent correctly to the apical junctions (Fig. 2.5F-G), none of these chimeric proteins is capable of rescuing $PATJ^{\Delta 1}$ (Fig. 2.5A). Notably, $PATJ_{\Delta L27\text{-}CR1(Baz)}$ and $PATJ_{\Delta L27\text{-}FERM(Yurt)}$ in contrast to $PATJ_{\Delta L27\text{-}PDZ(Sdt)}$ and $PATJ_{\Delta L27\text{-}PDZ(PAR6)}$ seem to be stabilized post-translationally (Fig. 2.5G). However, these elevated protein levels cannot be the reason for the lack of rescue capacity because expression of the chimeric proteins on a wild-type background does not result in increased lethality or obvious phenotypes as would be expected for dominant-negative proteins.

These results suggest that an association with both apical junctional complexes is essential for PATJ's function and that the targeting competence to these complexes is the most important feature of the L27 domain of PATJ.

Our data further indicate that a transient association with certain apical polarity protein complexes (Crb and Baz complex) rather than a direct targeting to the apical-junctional compartment is crucial for the function of PATJ during the development of *Drosophila*.

One possible explanation of these results is that the association of PATJ with both complexes, Baz-Sdt and Crb-Sdt is essential for PATJ's function. This might be

explained by the implication of PATJ in the regulation of the Actin/Myosin cytoskeleton: By modulating Myosin-phosphatase, PATJ regulates Myosin activity, which is essential for several morphological processes, including metamorphosis (Sen, Nagy-Zsver-Vadas et al. 2012). We furthermore showed that PATJ associates with Myosin in vivo by direct interaction with the Myosin regulatory light chain (MRLC, spaghetti-squashed, sqh in *Drosophila*)(Sen, Nagy-Zsver-Vadas et al. 2012). This is in line with results from mammalian PATJ, suggesting that mPATJ regulates Myosin-driven processes, such as cell migration and apical constriction (Shin, Wang et al. 2007; Ernkvist, Luna Persson et al. 2009; Nakajima and Tanoue 2011): mPATJ associates with the RhoA-GTPase exchange factor (GEF) Syx to control RhoA activity in lamellipodia, controlling the migration of endothelial cells (Ernkvist, Luna Persson et al. 2009). Another Rho-GEF (p114RhoGEF) was found to be recruited by mPATJ to regulate Myosin dynamics in the circumferential actomyosin belt during apical constriction (Nakajima and Tanoue 2011). Strikingly, aPKC modulates p114RhoGEF activity by phosphorylating the adaptor protein Lulu2. aPKC and its modulator PAR-6, in turn, have been shown to associate with both Baz/PAR-3 and Crb (Wodarz, Ramrath et al. 2000; Sotillos, Diaz-Meco et al. 2004; Harris and Peifer 2005). Thus the association of PATJ with both complexes might serve as a scaffold to assemble different Myosin-modulating proteins in a defined compartment of the cell.

PATJ might also function in different processes in the two distinct complexes which are localized to different cellular compartments: Baz associates with the AJ (Harris and Peifer 2005; Bulgakova, Grigoriev et al. 2013) which anchors Actin-Myosin filaments as well as Myosin-modulating enzymes (Shewan, Maddugoda et al. 2005; Yamada and Nelson 2007). Therefore Sdt-PATJ recruitment to the AJ would provide a mechanism for Myosin modulation as described before (Sen, Nagy-Zsver-Vadas et al. 2012). On the other hand, Crb has been described to link the Actin-cytoskeleton via the Moesin and $\beta_{\text{heavy-chain}}$ spectrin to the plasma membrane in a compartment between the AJ and the free apical membrane (Medina, Williams et al. 2002). Interestingly, control of Myosin and Moesin activity by (de)phosphorylation is accomplished by the

same set of enzymes (Fukata, Kimura et al. 1998; Oshiro, Fukata et al. 1998). Indeed, the Myosin Binding Subunit (MBS) of Myosin Phosphatase associates with both, the Baz-Sdt-PATJ and the Crb-Sdt-PATJ complex (Fig. 2.5J and K). Thus the association of PATJ with both apical complexes might be crucial to control (de-) phosphorylation of Myosin and Moesin during morphogenetic events in the development of *Drosophila*.

Chapter 2. PAR-6 stabilizes Sdt via Rpn13

PAR-6 prevents degradation of Stardust by inhibiting the proteasomal receptor Rpn13 in *Drosophila*

Rui Sun, Arnab Sen and Michael P. Krahn

This project aims to elucidate the role of PAR-6 in *Drosophila* epithelial polarity regulation. PAR-6 has been shown to interact with the Crb complex in previous studies with overexpression systems of cell lines and/or bacterially purified proteins. In current study, we established a new mechanism of PAR-6 in regulating epithelial polarity by inhibiting the degradation of Sdt via the proteasomal receptor Rpn13. It links protein quality control to the maintenance and establishment of cell polarity in *Drosophila*.

Author contributions:

Rui Sun: mostly the biochemistry studies and genetic studies, partially manuscript writing

Status:

Revision with ***Development***

PAR-6 prevents degradation of Stardust by inhibiting the proteasomal receptor Rpn13 in *Drosophila*

Epithelial cell polarity is one of the key prerequisites for the establishment of multicellular organisms and is frequently lost in epithelia-derived tumors. The conserved PDZ- and PB1-domain protein PAR-6 regulates apical-basal polarity and cell-cell junction formation in various epithelia. Here we show that in the *Drosophila* epidermis and cells of the follicular epithelium, PAR-6 modulates the stability of the Crumbs complex, which is a key regulator of the apical plasma membrane domain. PAR-6 associates with the proteasomal receptor Rpn13, thereby preventing the degradation of the Crumbs-adaptor protein Stardust. Loss of PAR-6 leads to degradation of Stardust and results in a disruption of apical-basal polarity, which can be rescued to some extent by downregulation of Rpn13. Notably, this new function of PAR-6 is independent of its well-described role as an activator of atypical protein kinase C. Thus, we identified a newly conserved mechanism of PAR-6 inhibiting proteasomal degradation of Stardust to maintain apical-basal polarity.

Keywords: cell polarity, *Drosophila*, Crb complex, proteasomal degradation

INTRODUCTION

One crucial hallmark of epithelial-derived tumors is the dedifferentiation of cells, which are reprogrammed to increase proliferation and downregulate cell-cell and cell-matrix adhesion. This process finally leads to an epithelial-to-mesenchymal transformation (EMT) and disintegration of single cells from the solid tumor and their dissemination (Wirtz, Konstantopoulos et al. 2011; Anchoori, Karanam et al. 2013; Lamouille, Xu et al. 2014). Strikingly, loss of apical-basal polarity occurs concomitantly with the down-regulation of cell-cell adhesion contacts, and several polarity regulators have been identified as tumor suppressors (Royer and Lu 2011). In *Drosophila* and mammalian epithelial cells, the conserved PAR complex defines, together with the Crumbs (Crb) complex, the apical plasma membrane domain and regulates apical-basal polarity as well as the formation of adherens junctions (AJ) and tight junctions (TJ) (Tepass 2012; Li, Mao et al. 2015).

The PAR complex consists of the scaffolding protein Bazooka(Baz)/PAR-3, the atypical Protein Kinase C (aPKC) and its adaptor/modulator protein PAR-6 (Suzuki and Ohno 2006).

PAR-6 functions as a modulator of aPKC kinase activity (Yamanaka, Horikoshi et al. 2001; Graybill, Wee et al. 2012) and recruits the small GTPases Rac1 and Cdc42 to the PAR-complex for activation, thus controlling AJ/TJ assembly, endocytosis and cytoskeleton rearrangements (Joberty, Petersen et al. 2000; Lin, Edwards et al. 2000; Qiu, Abo et al. 2000; Yamanaka, Horikoshi et al. 2001; Garrard, Capaldo et al. 2003; Hutterer, Betschinger et al. 2004; Georgiou, Marinari et al. 2008; Harris and Tepass 2008; Leibfried, Fricke et al. 2008; Zhang and Macara 2008; Georgiou and Baum 2010).

Within the Crb complex, the adaptor protein Stardust (Sdt, Pals1 in mammals) stabilizes the transmembrane protein Crb and links it to the Myosin-regulator PATJ (Pals1-associated TJ protein) (Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001; Roh, Makarova et al. 2002; Sen, Nagy-Zsver-Vadas et al. 2012; Sen, Sun et al.

2015). Loss of Sdt/Pals1 and Crb results in disturbed AJ/TJ (Tepass 1996; Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001; Roh, Fan et al. 2003; Straight, Shin et al. 2004; Fogg, Liu et al. 2005; Karp, Tan et al. 2008).

Several studies have demonstrated interactions between the PAR and the Crb complex facilitated by PAR-6: The PDZ domain of PAR-6 is capable to directly bind the C-terminus of Crb (Lemmers, Michel et al. 2004; Kempkens, Medina et al. 2006; Whitney, Peterson et al. 2016) as well as two evolutionary conserved motifs within the N-terminal region of Pals1 (Hurd, Gao et al. 2003; Gao and Macara 2004; Penkert, DiVittorio et al. 2004; Wang, Hurd et al. 2004). Consistently, in *PAR-6*-mutant *Drosophila* epithelia, Crb is mislocalized (Kempkens, Medina et al. 2006) and in mammalian cells, PAR-6 supports Pals1-dependent TJ formation (Hurd, Gao et al. 2003). However, as PAR-6 competes with Sdt/Pals1 for binding to Crb and loss of Sdt/Pals1 results in depletion of Crb from the membrane, it is unlikely that PAR-6 physically stabilizes the Crb-Sdt/Pals1 complex in order to ensure epithelial polarization.

Indeed we found in this study that, in the *Drosophila* epidermis, PAR-6 does not associate with Crb or Sdt at substantial levels. Instead, PAR-6 binds the proteasomal receptor Rpn13 and prevents the degradation of Sdt, independently of aPKC kinase activity. Sdt/Pals1 stabilization by PAR-6 seems to be evolutionarily conserved in mammalian cells.

EXPERIMENTAL PROCEDURES

Fly stocks and genetics. Germ line clones of a PAR-6 null allele ($PAR\text{-}6^{\Delta 226}$) (Petronczki and Knoblich 2001), an aPKC null allele ($aPKC^{k06403}$) (Wodarz, Ramrath et al. 2000), a Naz null allele $Baz^{815\text{-}8}$ were generated using the dominant female sterile technique. For immunoblots, embryos, which were homozygous mutant for $PAR\text{-}6^{\Delta 226}$, were identified using a fluorescent FM7a balancer (Abreu-Blanco, Verboon et al. 2011). For analysis of PAR-6 localization in *sdt*- or *crb* mutant embryos,

the following alleles were used: *sdt*^{K85} (Berger, Bulgakova et al. 2007) and *crb*^{11A22} (Tepass and Knust 1990). aPKC^{CAAX} (Sotillos, Diaz-Meco et al. 2004), UAS::GFP-Baz$_{S980A}$ (Krahn, Buckers et al. 2010) and Ubi::Sdt-GFP were expressed in *PAR-6*$^{\Delta 226}$ mutant follicle cell clones using tub::GAL4 and a FRT19A, tub::GAL80 allele (Bloomington *Drosophila* stock center at the University of Indiana). For downregulation of Rpn13 in PAR-6 mutant cells, the following lines were used: Trip.GL01155 (obtained from Bloomington and used to express in germ lines clones) and GD23874 and KK110471 (both obtained from VDRC stock center and used for expression in follicle cell clones). For MARCM (Mosaic analysis with repressible cell marker) clones in cells of the follicular epithelium, we used an aPKC null allele (*aPKC*^{k06403}) and the kinase-dead allele *aPKC*^{psu417} (Kim, Gailite et al. 2009). Generation of Ubi::Sdt-GFP and Ubi::Sdt$_{\Delta ECR1}$-GFP will be described elsewhere. Rpn13 knockout flies were generated using CRISPR/CAS9 technique with the targeting sequences listed in Suppl. Table 1.

Generation of antisera. Antisera against PAR-6 and Rpn13 were raised by injecting rabbits (AMSBIO, UK) with full-length recombinant GST-fusion proteins purified from *E.coli*. The specificity of the antisera was checked on PAR-6 and Rpn13 null alleles in Western Blotting and immunostainings.

Cell culture and transfections. DsRNA experiments in S2R cells were carried out as previously described (Krahn, Egger-Adam et al. 2009), using 20µg/ml dsRNA.

Immunoprecipitation and Western blotting. Immunoprecipitation and Western Blotting were carried out as previously described (Krahn, Egger-Adam et al. 2009) using the following antibodies: Rabbit anti Rpn13 (1:1000, this study), rabbit anti-Actin (1:2000, A2066, SIGMA), rabbit anti aPKC (aPKCζ, 1:500, Santa Cruz sc-216), rabbit anti-aPKC pT555 (aPKC autophosphorylation site, 1:200, Abcam #5813), rabbit anti-Baz (1:2000, Wodarz, Ramrath et al. 1999), rabbit anti-Baz pS980 (1:200, Krahn, Egger-Adam et al. 2009), mouse anti-Crb (Cq4, Developmental Studies Hybridoma Bank (DSHB), Tepass, Theres et al. 1990), mouse anti-GFP (1:500, sc-9996, Santa Cruz Inc.), mouse anti-myc (1:100, 9E10, DSHB), mouse anti-Pals1

(1:200, sc-365411, Santa Cruz Inc.), guinea pig anti-PAR6 (1:500, Kim, Gailite et al. 2009), rabbit anti-PAR-6 (1:2000, this study), guinea pig anti-PATJ (1:1000, Sen, Nagy-Zsver-Vadas et al. 2012), mouse anti-Sdt (1:20, Bulgakova, Rentsch et al. 2010), rabbit anti-PAR-6γ (1:250, sc-85097, Santa Cruz Inc.). Beads without antibody incubation served as a control in all experiments.

Immunohistochemistry. Embryos and egg chambers were fixed in 4% formaldehyde, phosphate buffer pH 7.4 as previously described. Primary antibodies used for indirect immunofluorescence were as follows: rabbit anti-Baz (1:2000, Wodarz, Ramrath et al. 1999), mouse anti-Crb (Cq4, DSHB, Tepass, Theres et al. 1990), rabbit anti-GFP (1:500, sc-8334, Santa Cruz Inc.), guinea pig anti-PAR6 (1:500, Kim, Gailite et al. 2009), guinea pig anti-PATJ (1:1000, Sen, Nagy-Zsver-Vadas et al. 2012), mouse anti-Sdt (1:20, Berger, Bulgakova et al. 2007). Secondary antibodies conjugated with Alexa 488, Alexa 568 and Alexa 647 (Life technology) were used at 1:400. Embryos at stage 9/10 were imaged. Images were taken on a Zeiss LSM 710 Meta confocal microscope and processed using Adobe Photoshop.

Quantitative PCR. For evaluation of mRNA level in wild-type embryos and embryos derived from $PAR6^{\Delta 226}$ germ line clones, 150 embryos were collected and dechorionized, and total RNA was extracted using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Residual genomic DNA was removed using RNase-free DNase (Qiagen). 500ng total RNA per sample were transcribed into cDNA using qScript cDNA synthesis kit (Quanta BioSciences) and quantitative PCR was carried out on a Light Cycler 480 (Roche) with SensiFast SYBR No-ROX kit (Bioline). For each reaction, 100ng cDNA were used. Data were analyzed with the $2^{-\Delta\Delta Ct}$ method. Data were normalized to a housekeeping gene expression (RP49). The student's t-test was used to compare the means, and differences were considered significant at $p<0.05$. The oligonucleotides used were listed in Suppl. Table 1.

RESULTS AND DISCUSSION

PAR-6 regulates the positioning of the Crb-Sdt complex - PAR-6 has been described to

be capable of directly associating with Crb as well as with Sdt/Pals1 (Hurd, Gao et al. 2003; Gao and Macara 2004; Lemmers, Michel et al. 2004; Penkert, DiVittorio et al. 2004; Wang, Hurd et al. 2004; Kempkens, Medina et al. 2006). Furthermore, the canonical binding partners of PAR-6, aPKC and Baz/PAR-3 interact with Crb (Sotillos, Diaz-Meco et al. 2004) and Sdt (Krahn, Buckers et al. 2010; Sen, Sun et al. 2015), respectively. Consequently, epithelial cells with impaired expression of PAR-6 display a mislocalization of Crb (Kempkens, Medina et al. 2006) and Pals1 (Hurd, Gao et al. 2003). We confirmed that in *Drosophila* PAR-6-mutant epithelial cells of the embryonic epidermis and of the follicular epithelium, Crb and PATJ are mislocalized, whereas the staining of Sdt is almost absent (Fig. 3.1B-D, compared to wild-type epithelium in A; note that cell morphology and polarity of the embryonic epidermis are strongly impaired upon the loss of PAR-6, Suppl. Fig. 3.1A-B). Notably, Baz still accumulates in distinct spots at the cell-cell contacts, suggesting that Baz functions upstream of PAR-6 in epithelial polarization (Fig. 3.1B-D), whereas apical-junctional localization of aPKC is lost in PAR-6 mutant cells (Suppl. Fig. 3.1B), indicating that PAR-6 is essential to target aPKC to the apical cell-cell contacts. Conversely, PAR-6 and Baz initially localize correctly (although to a lesser extent) to the apical junctions in epithelial cells of the epidermis of *sdt* or *crb*-mutant embryos (Suppl. Fig. 3.1C-D). Taken together these data confirm that PAR-6 regulates the localization of Crb/Sdt but can localize to the apical junctions independently of these proteins.

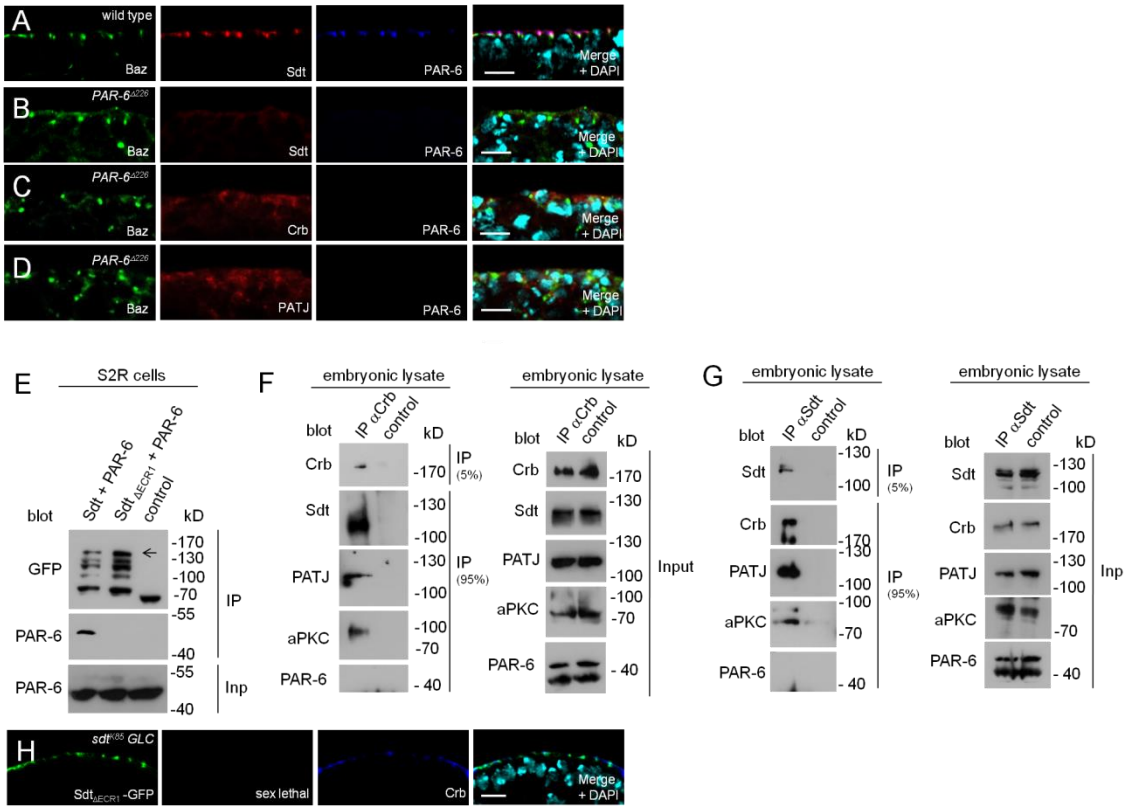


Figure 3.1 PAR-6 controls the localization of the Crb-Sdt complex. (A) PAR-6, Sdt, and Crb colocalize at the apical junctions in wild-type epithelial cells of the embryonic epidermis. (B-D) Sdt protein is absent in epidermal cells of PAR-6□226 mutant embryos (B), whereas Baz accumulates in spots at the cell-cell contacts (B and C), although cell morphology/polarity is strongly impaired. Crb and PATJ staining are cytoplasmic in PAR-6 deficient epithelia (C, D). (E) PAR-6-myc coimmunoprecipitates with Sdt-GFP but not with Sdt□ECR1-GFP in lysates from transfected S2R cells. The arrow shows the full-length proteins. (F-G) PAR-6 fails to coimmunoprecipitate with Crb (F) and Sdt (G) from wild-type embryonic lysates, whereas aPKC and PATJ associate with both. (H) Sdt□ECR1-GFP, expressed from a ubiquitous promoter in a sdtK85-mutant embryo, localizes correctly and is capable of recruiting Crb to the apical junctions. Scale bars = 5µm.

PAR-6 affects the stability of Sdt - Previous studies suggest that apart from the described Crb-Sdt-PATJ complex, Baz is capable of recruiting Sdt and PATJ to the apical junctions before the onset of Crb expression and that the Baz-Sdt-PATJ complex is preserved in later embryonic stages parallel to the Crb-Sdt-PATJ complex (Krahn, Buckers et al. 2010; Sen, Sun et al. 2015). Thus we tested whether PAR-6 supports the stability of a Baz-Sdt-PATJ or Crb-Sdt-PATJ complex by physically associating with these proteins.

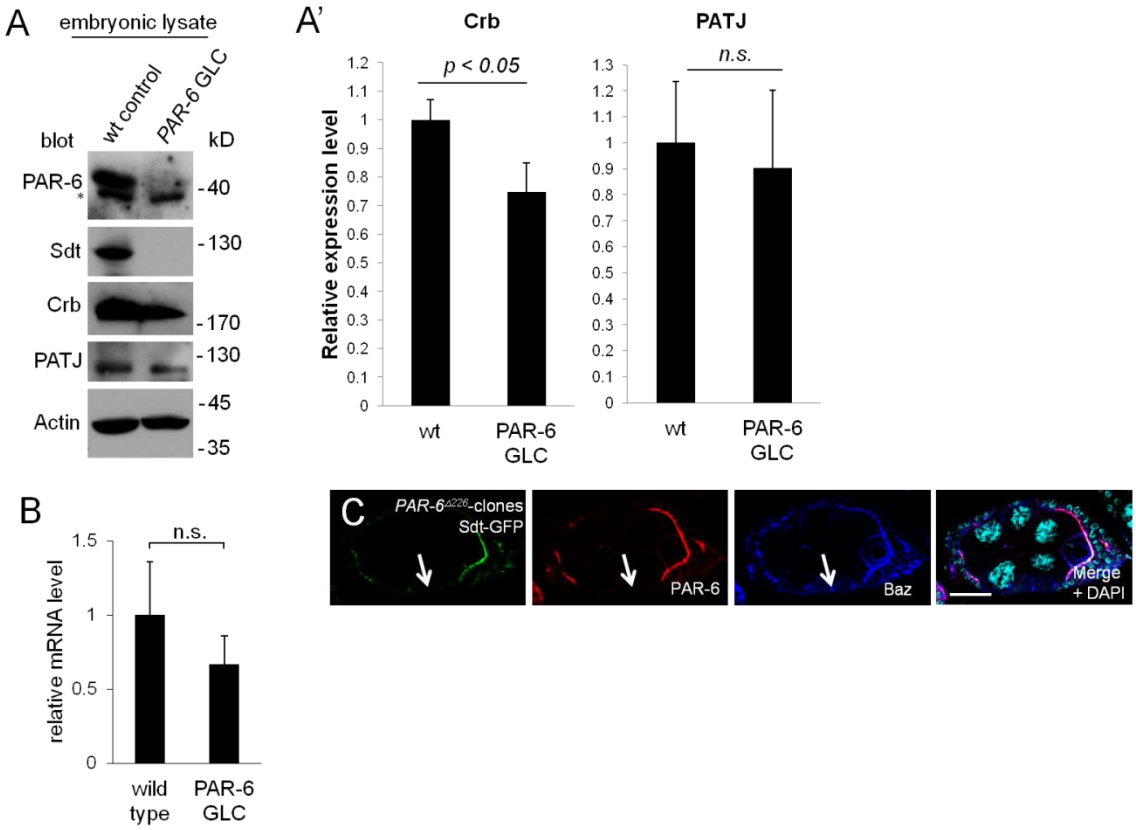


Figure 3.2 PAR-6 prevents degradation of Sdt. (A) Western Blot analysis of maternally and zygotically mutant PAR-6□226 embryos in comparison to wild-type embryos. The band marked with an asterisk is not specific to PAR-6. The protein expression levels of Crb and PATJ in three independent experiments were normalized to actin and quantified using Image J. Results are showing in A'. (B) Quantitative PCR of Sdt mRNA expression normalized against RP49 as house-keeping gene demonstrates that the mRNA of Sdt in PAR-6□226-mutant embryos is not significantly downregulated in comparison to wild-type embryos. mRNA expression of wild-type embryos was set as 1. Error bars represent standard derivation (SD) from six independent samples in each group. (C) Sdt expressed from a ubiquitous promoter is displaced from apical junctions in PAR-6 mutant cells of the follicular epithelium (arrow). Scale bar = 20µm.

In line with previous studies that used recombinant proteins or proteins overexpressed in cell culture experiments (Hurd, Gao et al. 2003; Penkert, DiVittorio et al. 2004; Wang, Hurd et al. 2004; Kempkens, Medina et al. 2006), we found PAR-6 co-immunoprecipitated with Sdt but not with Sdt lacking the evolutionary conserved region 1 (ECR1) from lysates of transfected *Drosophila* Schneider-2R (S2R) cells

(Fig. 3.1E). However, we were not able to detect a substantial association of PAR-6 with Crb or Sdt under endogenous conditions in embryonic lysates, whereas aPKC and PATJ co-immunoprecipitated with Crb and with Sdt (Fig. 3.1F and G). Furthermore, deletion of ECR1 ($Sdt_{\Delta ECR1}$) does not impair the localization of the mutant protein or Crb stabilization at the apical junctions in a *sdt*-mutant background (Fig. 3.1H). Thus it is unlikely that PAR-6 controls the assembly of the Crb-Sdt complex in the embryonic epidermis by the formation of a stable complex with these proteins.

aPKC kinase activity is not essential for PAR-6 function in Sdt stabilization - Because phosphorylation of Baz by aPKC results in a disassembly of the Baz-Sdt complex (Krahn, Buckers et al. 2010), we next tested whether the loss of PAR-6 results in a disturbed activity of aPKC. Several reports demonstrate that PAR-6 activates (Yamanaka, Horikoshi et al. 2001; Graybill, Wee et al. 2012) or inhibits (Atwood, Chabu et al. 2007) aPKC kinase activity, presumably depending on the cellular context. We found in S2R cells that the loss of PAR-6 abolished the autophosphorylation of endogenous aPKC, reflecting a strong decrease in its kinase activity (Suppl. Fig. 3.2A). Notably, the protein expression of aPKC was lower than in control-treated cells, indicating that PAR-6 might either enhance the expression of aPKC or stabilize the protein. However, overexpression of a constitutively active variant of aPKC (Sotillos, Diaz-Meco et al. 2004) in PAR-6 mutant follicle cells does not rescue Sdt localization (Suppl. Fig. 3.2B), indicating that activated aPKC cannot compensate the loss of PAR-6 with respect to Sdt stabilization.

Loss of aPKC in the embryonic epidermis (Suppl. Fig. 3.2C) or in the follicular epithelium (Suppl. Fig. 3.2D) results in loss of PAR-6 and Sdt from the junctions. In contrast, in cells homozygous for a kinase-dead aPKC allele (Kim, Gailite et al. 2009) PAR-6 and Sdt are correctly localized (Suppl. Fig. 3.2E), indicating that the kinase activity of aPKC is not essential for stabilizing PAR-6 at the apical junctions to ensure Sdt localization.

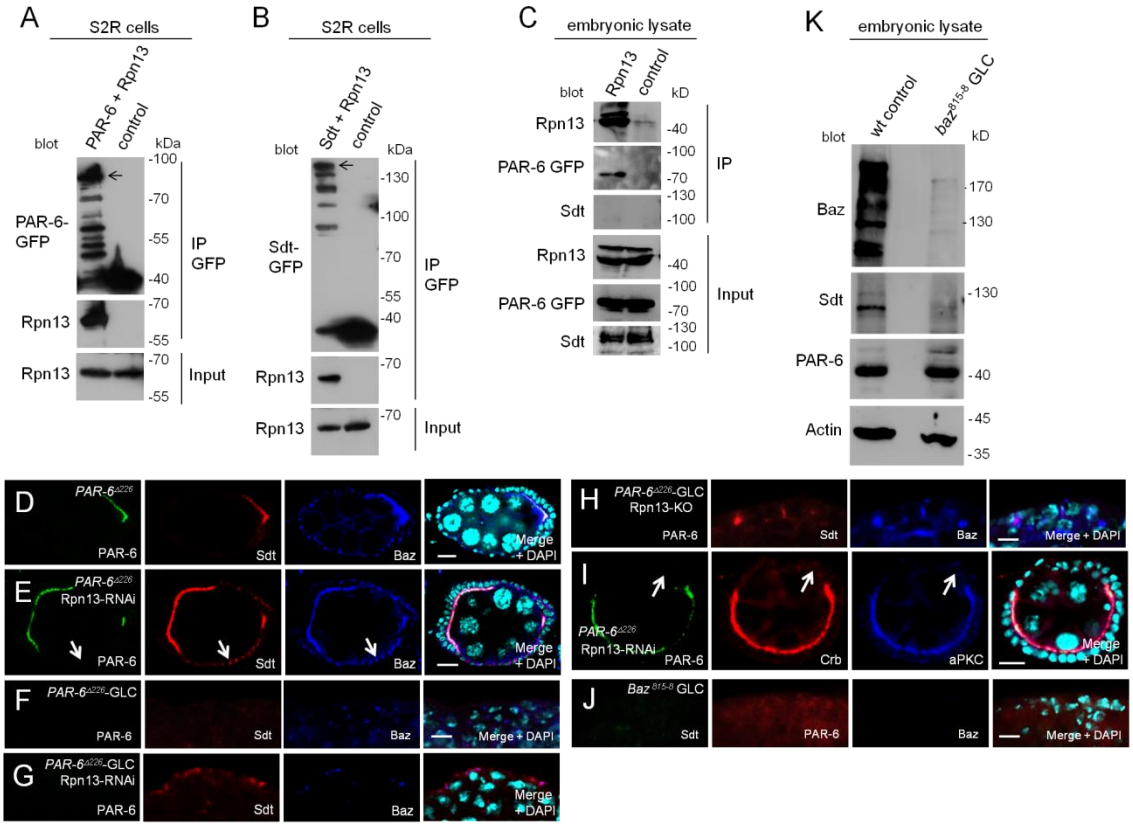


Figure 3.3 PAR-6 regulates Sdt stability via Rpn13. (A-B) Myc-Rpn13 coimmunoprecipitates with PAR-6-GFP (A) and Sdt-GFP (B) from lysates of transfected S2R cells. Arrows show the full-length proteins. (C) PAR-6-GFP (expressed from its endogenous promoter, Petronczki and Knoblich 2001) but not endogenous Sdt co-immunoprecipitates with endogenous Rpn13 from embryonic lysates. (D-H) Downregulation of Rpn13 restores Sdt localization at apical junctions in *PAR-6*$^{\Delta 226}$ mutant cells of the follicular epithelium (D shows PAR-6 mutant clones alone and E shows PAR-6 mutant clones with Rpn13-RNAi-Rescue) and the embryonic epidermis (F shows *PAR-6*$^{\Delta 226}$ GLC. G shows Rpn13 RNAi in the background of *PAR-6*$^{\Delta 226}$ GLC and H shows Rpn13 knock-out in *PAR-6*$^{\Delta 226}$ GLC). Note that cell morphology, apical-basal polarity and apical accumulation of Baz are still severely impaired upon loss of PAR-6. (I) Crb and aPKC junctional localizations are not rescued in *PAR-6*$^{\Delta 226}$ mutant follicle cells. (J) PAR-6 is mislocalized in *Baz* $^{815-8}$ GLC, while Sdt staining is absent. (K) Immunoblotting demonstrating that Sdt is degraded in embryos derived from *baz*$^{815-8}$-mutant germ line clones, although PAR-6 protein level is not affected. Scale bars = 10µm in D, E and I, 5µm in F, G, H, and J.

Finally, expression of a mutant variant of Baz (Baz_{S980A}), which cannot be phosphorylated by aPKC and fails to disassemble from Sdt (Krahn, Buckers et al. 2010), does not result in restored Sdt accumulation at the apical junction in PAR-6 mutant follicle cells (Suppl. Fig. 3.2F). These data indicate that PAR-6 controls the

localization of Sdt independently of aPKC kinase activity but that aPKC is essential to physically anchor PAR-6 at the apical junction to ensure Sdt apical junctional targeting.

Sdt is degraded in PAR-6 mutant cells - Western Blot analysis of PAR-6 mutant embryos revealed that Sdt protein is almost entirely absent in embryos derived from *PAR-6*$^{\Delta 226}$ mutant germ line clones, whereas Crb and PATJ protein expression is comparable to wild-type or slightly decreased (Fig. 3.2A and A'). In contrast, quantitative PCR of Sdt mRNA expression in these embryos does not reveal significant changes at the transcriptional level (Fig. 3.2B). Furthermore, constitutive expression of Sdt (Ubi::Sdt-GFP) in *PAR-6* mutant follicle cells does not promote Sdt stability or junctional localization (Fig. 3.2C), suggesting that loss of Sdt protein expression is regulated posttranscriptionally. Thus PAR-6 selectively controls the stability of Sdt but not of Crb or PATJ.

Sdt and PAR-6 interact with the proteasomal receptor Rpn13 - In a Yeast-two-hydrid screen of PDZ-domain proteins in *C. elegans* (Lenfant, Polanowska et al. 2010), the proteasomal receptor Rpn13 was found to interact with the PDZ-domain of PAR-6. Rpn13 (also named Adhesion regulating molecule 1, Adrm1) functions as a receptor for ubiquitinylated proteins, enhancing their degradation (Hamazaki, Iemura et al. 2006; Jorgensen, Lauridsen et al. 2006; Qiu, Ouyang et al. 2006). In contrast to core components of the proteasome, loss of Rpn13 in yeast and mammalian cells has no or only subtle effects on overall protein polyubiquitination and degradation, indicating that it may rather function in the degradation of a certain subset of proteins (Hamazaki, Iemura et al. 2006; Jorgensen, Lauridsen et al. 2006; Qiu, Ouyang et al. 2006). Notably, Rpn13 mRNA levels are upregulated in several carcinoma cell lines and tumor samples (Simins, Weighardt et al. 1999; Carvalho, Postma et al. 2009; Chen, Hu et al. 2009; Fejzo, Anderson et al. 2013).

We confirmed that *Drosophila* PAR-6 interacts with Rpn13 in transfected S2R cells and under endogenous conditions in embryonic lysates (Fig. 3.3A and C). Rpn13 is also capable of co-immunoprecipitating with Sdt-GFP from lysates of transfected S2R

cells, but at endogenous protein levels, no association of Sdt and Rpn13 can be detected (Fig. 3.3B and C). Next, we tested whether PAR-6 modulates degradation of Sdt by binding to Rpn13 and thereby prevents Rpn13 from targeting Sdt to proteasomal degradation. Indeed, RNAi-mediated downregulation and CRISPR/CAS9 based knocking out of Rpn13 in *PAR-6*$^{\Delta 226}$ mutant embryos or cells of the follicular epithelium resulted in an accumulation of Sdt at cell-cell contacts, where Baz is localized (Fig. 3.3E compared to D and F/H compared to G). Since Rpn13 is able to enhance the protein polyubiquitination, we assume that this rescue effect by Rpn13 compromise might be because of the attenuated ubiquitylation of Sdt, therefore preserving a certain amount of protein level in the *PAR-6* $^{\Delta 226}$ mutant cells. In contrast, reduction of Rpn13 in *PAR-6* $^{\Delta 226}$ mutant cells does not restore Crb or aPKC junctional localization (Fig. 3.3I), suggesting that PAR-6 is essential for aPKC stability (Suppl. Fig. 3.2A), which in turn functions to stabilize Crb at the sub-apical region.

Finally, we investigated whether Baz controls Crb/Sdt localization by targeting PAR-6 to the apical junctions thus preventing degradation of Sdt. Indeed, the loss of Baz results not only in mislocalization of PAR-6 in the embryonic epidermis but leads to degradation of Sdt, whereas the overall protein amount of PAR-6 is not affected (Fig. 3.4J and K). These data provide an explanation for the described hierarchy in the polarization of the embryonic epidermal epithelium (Tepass 2012): Membrane-targeting of PAR-6 by Baz is essential for stabilizing Sdt, thus indirectly preserving the Crb-complex at the apical junctions.

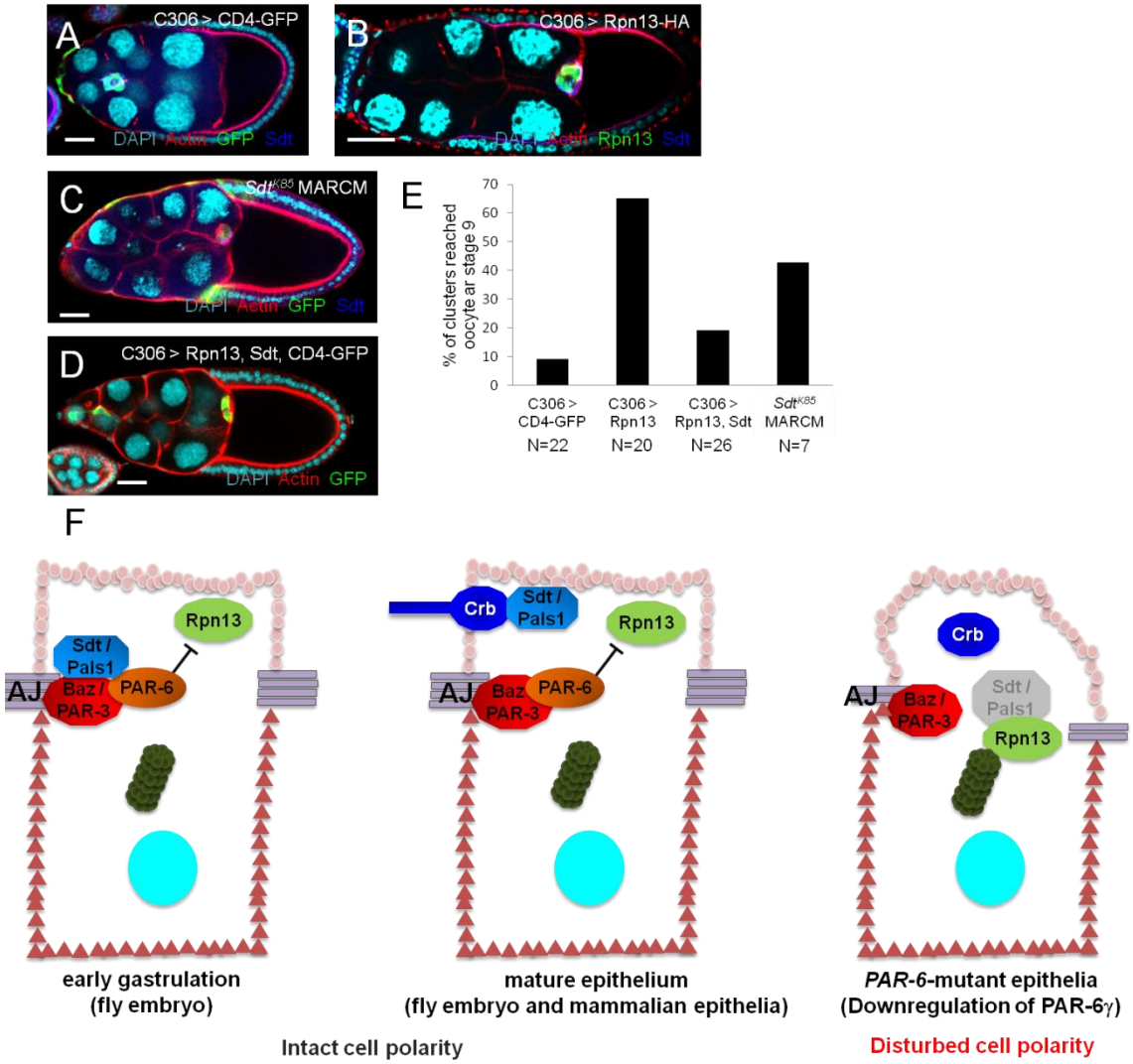


Figure 3.4 Rpn13 overexpression enhances border-cell collective migration, which can be balanced by co-overexpression of Sdt. (A-D) Stainings of egg chambers with indicated antibodies and fluorescent dyes with different genotypes. Scale bars = 20μM. (E) Quantification of border-cell clusters reached oocyte at stage 9. Rpn13 overexpression enhances the migration and co-overexpression of Sdt and Rpn13 balanced this effect to some extent. Mutant of Sdt increases the migrating ability. (F) Model of PAR-6 preventing Rpn13-mediated degradation of Sdt/Pals1 in epithelial cells. In early epithelial polarization of the embryonic epidermis (gastrulation), Baz recruits Sdt and PAR-6 to the apical cell-cell contacts. Upon Crb expression (from stage 8 onwards), Sdt stabilizes Crb at the apical tip of the lateral membrane. In the absence of PAR-6, Rpn13 binds and degrades Sdt, resulting in a loss of apical-junctional Crb and a disturbed apical-basal polarity.

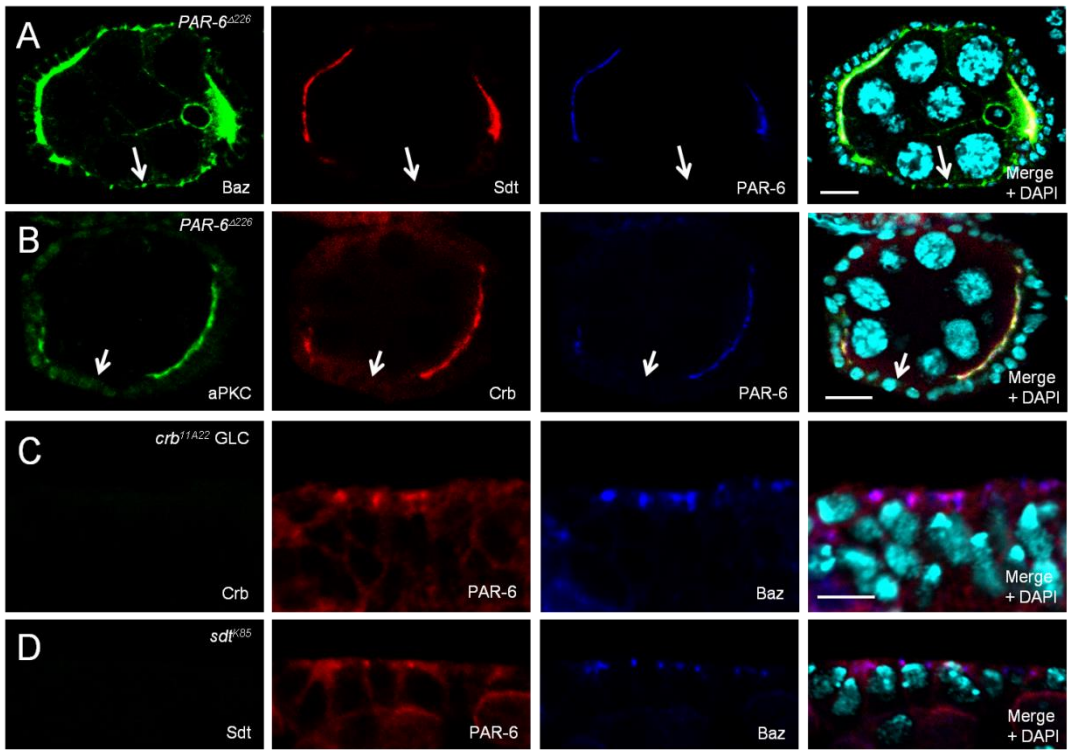
Rpn13 overexpression enhances border-cell collective migration, which can be balanced by co-overexpression of Sdt - Cell polarity proteins are intensively showed

to be involved in the cell migration during various contexts (Etienne-Manneville 2008; Muthuswamy and Xue 2012). In *Drosophila*, border cells collective migration has been proven to be a good *in vivo* model to study this issue (Montell 2003). In *Drosophila* egg chambers, the anterior pair of polar cells recruits several adjacent follicular cells to form the border-cell cluster and this cluster will delaminate and migrate through nursing cells, reaching the anterior side of the oocyte by stage 10. The previous study shows that PAR-6 and Baz are required for proper border-cell collective migration (Pinheiro and Montell 2004). Taking advantage of the MARCM technique, we generated homozygous *Sdt* mutant border-cell cluster clones. Clusters lacking Sdt displayed increased migration ability with approximately 43% reached the oocytes in stage 9 egg chambers while the control was only 9% (Fig. 3.5C and E).

Rpn13 also named as adhesion regulating molecule 1 (Adrm1), is found to be overexpressed in multiple cancer specimens and is proposed as a novel target for cancer therapy (Anchoori, Karanam et al. 2013; Song, Ray et al. 2016). Knocking down or inhibition of Rpn13 leads to decreased cell migration, cell proliferation and tumorigenicity in cells deriving from different cancers (Song, Das et al. 2014; Zheng, Guo et al. 2015; Song, Ray et al. 2016). To test the role of Rpn13 in cell migration, we overexpressed it in *Drosophila* border cells and the adjacent polar cells under a C306 promoter. Overexpression of Rpn13 resulted in 65% border-cell clusters reached oocytes in the stage 9 egg chambers (Fig. 3.5B, D, and E). This enhanced collective migration was balanced by co-overexpression of Sdt under the same promoter. As showing in Fig. 3.5E, less than 20% of clusters reached the oocytes. Taken together, these data indicate that the genetic interplay between polarity cues and Rpn13 is of great importance during cell migration.

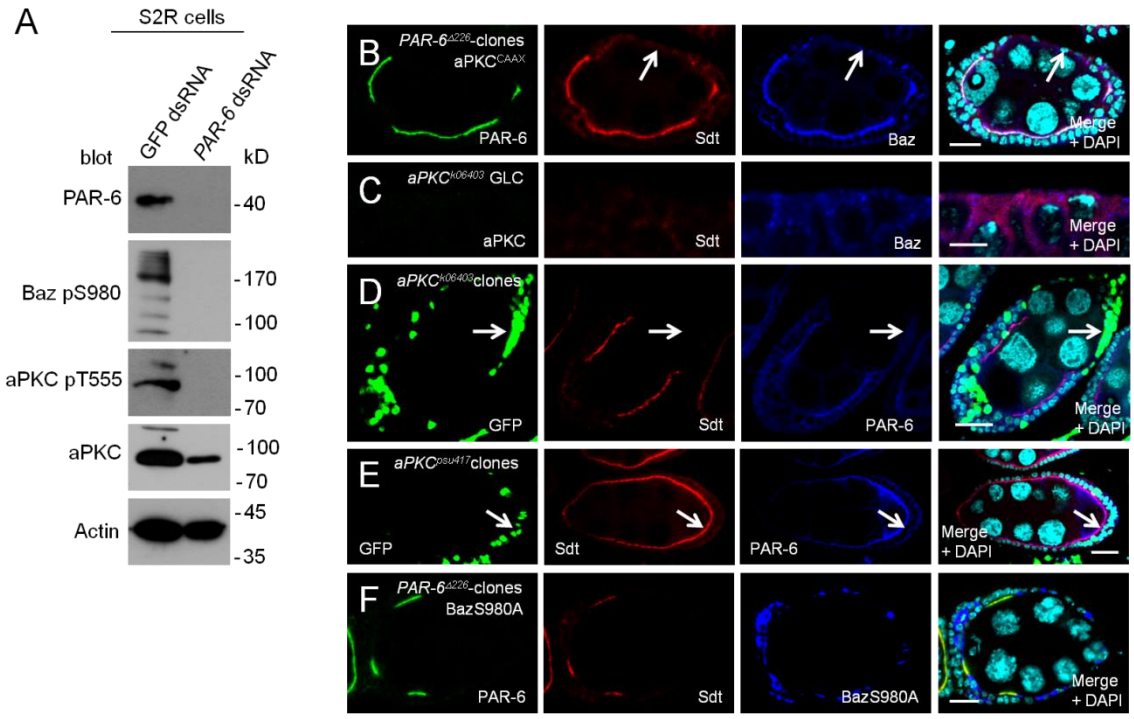
Taken together our data suggest a model in which PAR-6 controls the function of the Crb-complex in epithelial cells by preventing the proteasomal degradation of Sdt/Pals1, which in turn stabilizes Crb at the apical cell-cell contacts (Figure 3.5). This process is selective for Sdt/Pals1 whereas other polarity proteins (e.g., Crb and PATJ) are not affected or only slightly degraded upon loss of PAR-6. Baz/PAR-3

functions to position PAR-6 at the apical junctions (TJ in vertebrates) in order to establish a micro-compartment for PAR-6 mediated Rpn13-inhibition, thus preventing Sdt/Pals1 degradation (Figure 3.5).



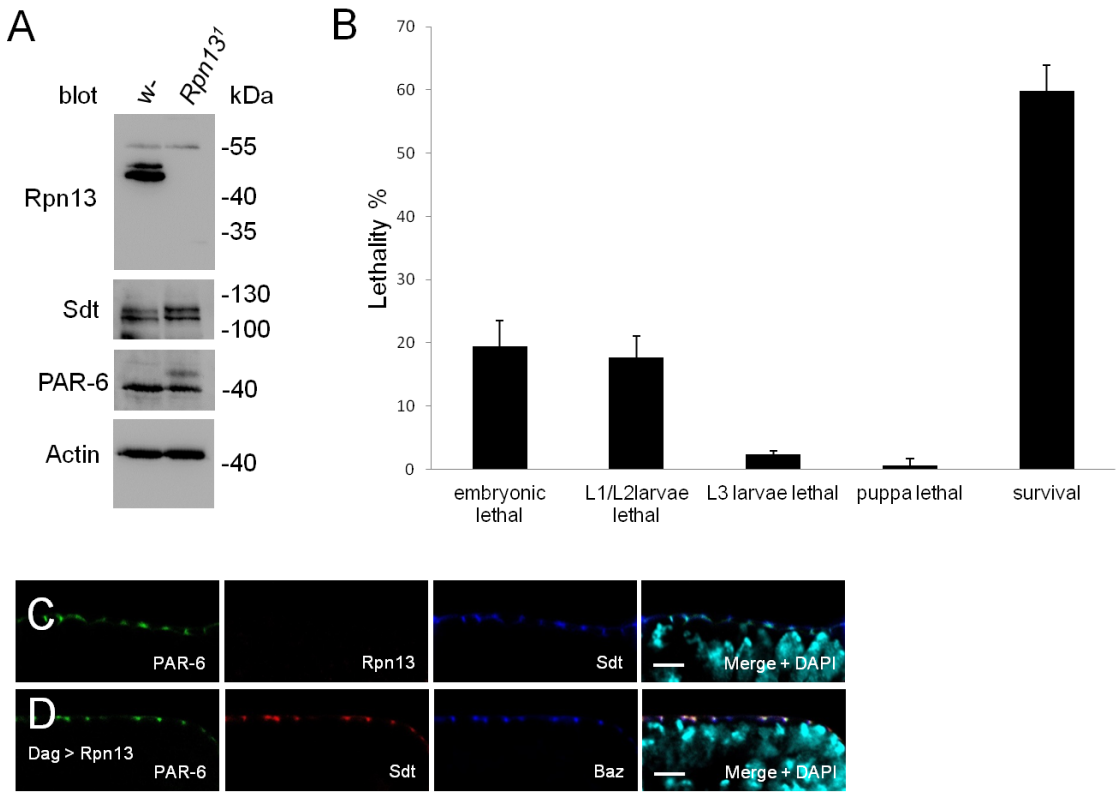
Supplemental Figure 3.1 PAR-6 localization at the apical junctions in epithelial cells of the embryonic epidermis does not depend on Crb or Sdt. (A-B) PAR-6 mutant cells of the follicular epithelium display a loss of Sdt (A), Crb and aPKC (B) at the apical cell-cell contacts, whereas Baz is still localized although strongly reduced (A, arrow, note that exposure for Baz was upregulated to visualize residual staining). (C-D) Zygotic mutant embryos for *crb*^{11A22} (C) and *sdt*^{K85} (D) were analyzed for PAR-6 and Baz localization at embryonic stage 9. Scale bars = 10µm in A and B, 5µm in C and D.

This mechanism is conserved from fly to mammals. Given the fact that Rpn13 has been reported to be upregulated in several carcinoma cell lines and tumor samples (Simins, Weighardt et al. 1999; Carvalho, Postma et al. 2009; Chen, Hu et al. 2009; Fejzo, Anderson et al. 2013), it might function to degrade Pals1 in these transformed cells, thereby destabilizing the cell-cell contacts and promoting tumor growth and metastasis.



Supplemental Figure 3.2 PAR-6-dependent activation of aPKC does not contribute to Sdt stabilization. (A) Downregulation of PAR-6 in S2R+ cells results in decreased aPKC expression and an abolished aPKC activity (aPKC pT555 = autophosphorylation site) and Baz phosphorylation (Baz pS980). (B) Expression of a constitutively active aPKC-variant does not rescue the loss of Sdt in *PAR-6* $^{\Delta 226}$ mutant follicle cells. (C-D) In embryos and follicle cells which are mutant for an aPKC-null allele (*aPKC*^{k06403}), Sdt and PAR-6 are displaced from the apical junctions. (E) A kinase-dead aPKC-allele (*aPKC*^{psu417}) does not exhibit defects in PAR-6 or Sdt localization. Homozygous mutant cells were marked by nuclear GFP expression in D and E. (F) Sdt localization is not rescued by expression of BazS980A in *PAR-6* $^{\Delta 226}$ mutant follicle cells. Scale bars = 5µm in C, 20µm in B, D, and E, 10µm in F.

Notably, our data reveal a new role of PAR-6 in the regulation of apical-basal polarity, which is independent of its described function in enhancing the kinase activity of aPKC (Yamanaka, Horikoshi et al. 2001; Graybill, Wee et al. 2012), but requires aPKC as the (scaffolding) binding partner, as suggested before (Kim, Gailite et al. 2009).



Supplemental Figure 3.3 Rpn13 mutant flies are viable but male sterile. (A) Western Blotting of wild-type and Rpn131-mutant flies. (B) Lethality test of Rpn131-mutant embryos. (C-D) PAR-6 and Sdt are normally localized in the Rpn131-mutant embryo and the one with Rpn13 overexpression under a Daughterless promoter.

In the study we showed that Sdt/Pals1 is, apart from a particular isoform of the transcription factor p63, liver kinase B1 (LKB1) and inducible nitric oxide synthase (iNOS), another important target of Rpn13 (Huang and Ratovitski 2010; Huang and Ratovitski 2010; Mazumdar, Gorgun et al. 2010). These findings raise the question that whether Rpn13 regulates a certain subgroup of proteins (e.g. cell polarity/junction-associated proteins). This is of high importance, in particular in the context of Rpn13 being upregulated in various types of cancer (Simins, Weighardt et al. 1999; Pilarsky, Wenzig et al. 2004; Carvalho, Postma et al. 2009; Chen, Hu et al. 2009; Fejzo, Anderson et al. 2013). Strikingly Rpn13 knock-out mice (Al-Shami, Jhaver et al. 2010) and flies (Supplemental Figure 3.3) are viable and show (apart from male sterility) no obvious phenotypes. This renders the possibility of targeting Rpn13 in chemotherapy (Anchoori, Karanam et al. 2013; Song, Ray et al. 2016) with

reduced side-effects to prevent Pals1-degradation, cell-cell junction disassembly, tumor growth and metastasis of cancer cells.

Chapter 3. Sdt structure-function analysis

Domain-specific functions of Stardust in *Drosophila* embryonic development

Leonie Koch, Sabine Feicht, Rui Sun, Arnab Sen and Michael P. Krahn

This project aims to reveal the protein-interaction features of Sdt *in vivo*. Several genetically modified flies harboring different domain-deletion variants of Sdt were generated and the detailed phenotypes were analyzed. We confirmed the important function of PDZ domain of Sdt *in vivo*. Additionally, the unexpected roles of GUK and L27N domains in epithelial polarity regulation were also identified.

Author contributions:

Rui Sun: mostly the biochemistry studies and partially genetic studies

Status:

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Domain-specific functions of Stardust in *Drosophila* embryonic development

In *Drosophila*, the adaptor protein Stardust is essential for the stabilization of the polarity determinant Crumbs in various epithelial tissues, including the embryonic epidermis, the follicular epithelium and photoreceptor cells of the compound eye. In turn, Stardust recruits another adaptor protein, PATJ, to the subapical region to support adherens junction formation and morphogenetic events. Moreover, Stardust binds to Lin-7, which is dispensable in epithelial cells but functions in postsynaptic vesicle fusion. Finally, Stardust has been reported to bind directly to PAR-6, thereby linking the Crumbs-Stardust-PATJ complex to the PAR-6/aPKC-complex. PAR-6 and aPKC are also capable to directly bind Bazooka (the Drosophila homolog of PAR-3) to form the PAR/aPKC complex, which is essential for apical-basal polarity and cell-cell contact formation in most epithelia. However, little is known about the physiological relevance of these interactions in the embryonic epidermis of *Drosophila* in vivo. Thus we performed a structure-function analysis of the annotated domains with GFP-tagged Stardust and evaluated the localization and function of the mutant proteins in epithelial cells of the embryonic epidermis. The data presented here confirm a crucial role of the PDZ domain in binding Crumbs and recruiting the protein to the subapical region. However, the isolated PDZ domain is not capable of being recruited to the cortex, and the SH3 domain is essential to support the binding to Crumbs. Notably, the conserved N-terminal regions (ECR1 and ECR2) are not crucial for epithelial polarity. Finally, the GUK domain plays an important role in the protein's function, which is not directly linked to Crumbs stabilization, and the L27N domain is essential for epithelial polarization independently of recruiting PATJ.

Keywords: Crumbs, Stardust, PAR-6, Drosophila, epithelial polarity

INTRODUCTION

Apical-basal polarity is one of the most important characteristics of many epithelial cells to accomplish their function. In these cells, the apical plasma membrane domain faces an organ lumen or the outer surface of an organism on one side, and the basal plasma membrane domain connects the epithelial cells with the basal membrane and connective tissue on the other side. Furthermore, the apical-basal polarity is closely linked to the formation of cell-cell contacts, thus ensuring the mechanical integrity of the tissue as well as regulation of a paracellular diffusion barrier.

Many polarity determinants which regulate apical-basal polarity and junction assembly are highly conserved throughout evolution, from worm to human. According to their localization and function, polarity proteins can be classified as apical polarity regulators (APRs) or basolateral polarity regulators (BLPRs) (Tepass 2012).

Apical and basolateral polarity proteins interact in an antagonistic way, using negative feedback mechanisms. Thus, they assure the differentiation of the plasma membrane in an apical and a basolateral domain, which is the prerequisite for correct sorting, e.g., of channels, receptors, enzymes or lipids.

In *Drosophila*, two apical protein complexes determine the apical plasma membrane domain – partly in redundancy, depending on the tissue and developmental context: the PAR-complex and the Crumbs-complex. The PAR-complex is composed of the serine-threonine-kinase aPKC (atypical Protein Kinase C), the scaffold proteins PAR-6 and Bazooka (Baz) and the GTPase Cdc42 (Suzuki and Ohno 2006; Nance and Zallen 2011).

The second apical polarity complex, the Crumbs-complex, consists in its canonical form of the transmembrane protein Crumbs (Crb), the adaptor protein Stardust (Sdt; Protein Associated with Lin-7 One, Pals1 in mammals), the PDZ domain-containing protein PATJ (Pals1-associated-tight-junction-protein) and Lin-7 (Bulgakova and Knust 2009).

Crb is a key determinant of apical identity: Loss of Crb in the embryonic epidermis results in a strong reduction of the apical domain (reflected by an impaired secretion of the cuticle) and a weakening of the adherens junctions (AJ) (Tepass, Theres et al. 1990; Tepass 1996), and vice versa, overexpression of Crb enlarges the apical plasma membrane at the expense of the basolateral domain (Wodarz, Hinz et al. 1995). Notably, loss of Crb is at least partly compensated by the simultaneous reduction of one of the basolateral polarity cues (Lethal (2) Giant Larvae, Lgl, Discs Large or Scribble) (Tanentzapf and Tepass 2003).

The big extracellular domain of Crb has been reported to facilitate a homophilic interaction (Letizia, Ricardo et al. 2013), although it might be dispensable for determining the apical identity (Wodarz, Hinz et al. 1995; Klebes and Knust 2000; Letizia, Ricardo et al. 2013). In contrast, the short – highly conserved – intracellular tail contains two important protein interaction domains: The C-terminal ERLI motif facilitates binding to Sdt which is in turn necessary to stabilize Crb in the membrane at the subapical region (Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001). Furthermore, PAR-6 competes with Sdt for binding the ERLI-motif of Crb *in vitro* (Lemmers, Michel et al. 2004; Kempkens, Medina et al. 2006). Secondly, Crb is linked via the FERM (Protein 4.1 – Ezrin/Radixin/Moesin) domain to the subcortical Actin cytoskeleton through the FERM domain protein Moesin and β_{heavy}-spectrin (Medina, Williams et al. 2002; Wei, Li et al. 2015). Another function of the FERM domain is binding to Yurt, which restricts Crb activity (Laprise, Beronja et al. 2006). Apart from the control of apical-basal polarity, Crb controls cell proliferation and organ growth by influencing the Hippo-pathway via binding to the FERM domain protein Expanded (Chen, Gajewski et al. 2010; Grzeschik, Parsons et al. 2010; Robinson, Huang et al. 2010) and by restricting the activation of Notch (Richardson and Pichaud 2010).

As Sdt stabilizes Crb at the correct subcellular position, *sdt* alleles exhibit similar phenotypes to *crb* mutants regarding apical-basal polarity (Tepass and Knust 1993; Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001; Lin, Currinn et al.

2015)

However, Sdt is a large scaffold protein exhibiting several protein-interaction domains (Fig. 1A): The N-terminal evolutionary conserved regions (ECR) 1 and 2 have been reported to be involved in the binding of mammalian Pals1 to two different PAR-6 proteins in cultured mammalian cells (Hurd, Gao et al. 2003; Gao and Macara 2004; Wang, Hurd et al. 2004). However, apart from the function of a large N-terminal fragment, including ECR1/2, in the apical recruitment of PAR-6 in photoreceptor cells (Bulgakova, Kempkens et al. 2008), no function of this interaction in epithelia *in vivo* has been reported until now.

The first L27 (Lin-2/Lin-7) domain (L27N) is essential to recruit PATJ to the subapical region (Penalva and Mirouse 2012; Sen, Sun et al. 2015), which in turn stabilizes the AJ by promoting Myosin activity (Sen, Nagy-Zsver-Vadas et al. 2012) but is not essential to stabilize the Crb-Sdt complex in the embryonic epidermis (Penalva and Mirouse 2012; Sen, Nagy-Zsver-Vadas et al. 2012; Zhou and Hong 2012). Via its second L27 domain (L27C), Sdt recruits Lin-7 (Veli) to the plasma membrane, which is not crucial for epithelial polarity in *Drosophila* but functions at the postsynaptic synapse membrane to prevent light-induced photoreceptor degeneration (Bachmann, Timmer et al. 2004; Soukup, Pocha et al. 2013). The PDZ domain of Sdt binds not only to the ERLI-motif of Crb but also to a conserved region of Baz (Krahn, Buckers et al. 2010), thus establishing two different polarity complexes which are present in parallel in the embryonic epidermis throughout embryogenesis: the Crb-Sdt-PATJ and the Baz-Sdt-PATJ complex (Sen, Sun et al. 2015).

Apart from the PDZ domain, recent data from the crystallization of parts of the Crb-Pals1 complex reveal an important role of the SH3 (Src-homology-3) and GUK (guanylate kinase) domains in stabilizing the Crb-Pals1 complex, thus regulating lumen formation in mammalian cysts models (Li, Wei et al. 2014).

Because limited information is available about the function of the protein-interaction

domains of Sdt *in vivo*, we performed a structure-function analysis of Sdt in the embryonic epidermis of *Drosophila*.

EXPERIMENTAL PROCEDURES

Plasmids. The ORF of Sdt-F (formerly described as Sdt-B1) was cloned into pENTR (life technology). Deletions of the distinct domains (introducing a flexible 3xGlycin spacer instead of the domain) were established by mutagenesis PCR using the following oligonucleotides:

SdtDECR1-F: 5'- CAAGATAACGGTCCAGGTGGAGGTGACACGTTCATCGCA-3' – deletion of aa11-19;

SdtDECR2-F: 5'-TACCAGGAGCAACTGGGAGGTGGAGAGCGCATAGCGCAG-3' – deletion of aa119-125;

SdtDL27N-F: 5'- GCGGAACAGATCGATGGTGGAGGTTCTGGTCCACTGCAT-3' – deletion of aa193-250;

SdtDL27C-F: 5'- CGCGTCTCTGGTCCAGGAGGTGGAGGCACGCCCTCGCCA-3' – deletion of aa254-304;

SdtDPDZ-F: 5'-ATCATCCAGATCGAGGGAGGTGGACCAGCGGGTAGTCCA-3' - deletion of aa405-476;

SdtDSH3-F: 5'-GACACCGCCGTGTTGGGAGGTGGACAGTCGTTCCAGCAT-3' – deletion of aa509-568;

SdtDGUK-F: 5'- GCTACCCACAAGCGGGGAGGTGGACAATGGGTGCCCGCC-3' – deletion of aa657-841;

The ORF of Sdt variants was subcloned into UWGattB (modified from UWG, which was obtained from the Drosophila Genomic Resource Center as described before, Sen, Sun et al. 2015) vector by clonase reaction (life technology).

Fly stocks and genetics. Fly stocks were cultured on standard cornmeal agar food and maintained at 25°C. Transgenic flies of UAS::Sdt-GFP and the Ubi::Sdt-GFP variants

were established using the Phi-C31-Integrase system (Groth, Fish et al. 2004) with attP40 (for the UAS-construct) and attPVK00037 (22A, for the Ubi::Sdt-GFP-constructs). En::GAL4 and arm::GAL4 were obtained from the Bloomington stock center. The sdt^{K85} allele (Berger, Bulgakova et al. 2007) was used for evaluation of the function of (mutant) Sdt proteins *in vivo* by producing germ line clones with the female sterile OvoD technique (Chou, Noll et al. 1993) using FRT19A-OvoD1, hs::Flp (BL#23880). Females of sdt^{K85}, FRT19A/OvoD1, FRT19A; Ubi::Sdt-GFP were mated with males carrying an FM7-ChFP-fluorescent balancer (Abreu-Blanco, Verboon et al. 2012) and Ubi::Sdt-GFP. Embryos which were homozygous for sdt^{K85} were identified using staining against sex lethal (Bopp, Bell et al. 1991) for immunostainings and by sorting against ChFP for cuticle preparations and lethality tests. The crb^{11A22} allele (Jürgens, Wieschaus et al. 1984) was used for cuticle preparations of *crb*-mutant embryos. For lethality tests, sdt^{K85}-mutant embryos expressing the Sdt protein variants were generated as described above. In three independent experiments, 100 homozygous mutant embryos were scored (in each experiment) for embryonic lethality, L1/L2-, L3- and pupal lethality and surviving flies. Error bars indicate the standard error of the mean.

Generation of an antibody against Sdt. An antibody directed against the PDZ domain of Sdt was raised by immunizing rabbits with GST-Sdt_{PDZ} (Davids Biotechnology, Regensburg, Germany). The specificity of the serum was tested in immunostainings on sdt^{K85} mutant embryos (Suppl. Fig. 1C-D).

Immunoprecipitation and Western blotting. For immunoprecipitations, w^- embryos or embryos expressing GFP-tagged Sdt-variants from an overnight collection were dechorionated and lysed in lysis buffer (1% Triton X-100, 150mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 50mM TRIS-HCl pH 7.5) supplemented with protease inhibitors. After centrifugation, either mouse anti-Sdt antibody (Bulgakova, Rentsch et al. 2010), mouse anti β-Galactosidase or rabbit anti-GFP (Life Technologies) was added to embryonic lysate corresponding to 500 µg total protein. Immune complexes were harvested using protein A/G-conjugated agarose (BioVision). Beads were washed five

times in lysis buffer and boiled in 2x SDS sample buffer before SDS-PAGE and Western blot. Immunoprecipitation from Schneider 2R cells transfected with Sdt-GFP (Sdt UWGattB) and Ubi::PAR-6-myc (PAR-6 UWM) was similarly performed as described previously (Sen, Nagy-Zsver-Vadas et al. 2012). Western blotting was done according to standard procedures. The primary antibodies used for Western blotting were as follows: mouse anti Crb (Cq4, 1:50, DSHB), mouse anti Sdt (1:20, Bulgakova, Rentsch et al. 2010), guinea pig anti PATJ (1:1000, Sen, Nagy-Zsver-Vadas et al. 2012), rabbit anti-Baz (1:2000, Wodarz, Ramrath et al. 1999), rabbit anti aPKC (aPKCζ, 1:500, Santa Cruz sc-216), mouse anti-GFP (1:500, B2, Santa Cruz sc-9996) and guinea pig anti-PAR6 (1:500, Kim, Gailite et al. 2009).

Immunohistochemistry. Embryos were fixed in 4% formaldehyde, phosphate buffer pH 7.4 as previously described (Krahn, Egger-Adam et al. 2009). The primary antibodies used for indirect immunofluorescence were as follows: guinea pig anti PATJ (1:500, Sen, Nagy-Zsver-Vadas et al. 2012), mouse anti Sdt (1:20, Bulgakova, Rentsch et al. 2010), rabbit anti Sdt (1:2000, this study), rabbit anti-Baz (1:1000, Wodarz, Ramrath et al. 1999), mouse anti Crb (Cq4, 1:50, DSHB), mouse anti Dlg (1:50, DSHB), rabbit anti-GFP (#A11122, 1:1000, Life Technologies) and chicken anti-GFP (1:2000, Aves Laboratories). Secondary antibodies conjugated with Alexa 488, Alexa 568 and Alexa 647 (Life Technologies) were used at 1:400.

Images were taken on a Zeiss LSM 710 Meta confocal microscope and processed using Adobe Photoshop.

RESULTS AND DISCUSSION

Sdt exhibits several evolutionary conserved protein interaction domains - Similar to other MAGUK (Membrane-associated GUK) proteins, e.g., Discs large, ZO-1, Calcium/Calmodulin-dependent Serine protein Kinase or other members of the MPP (MAGUK p55 subfamily) family, Sdt contains a GUK domain, which is catalytically inactive but might facilitate protein-protein interaction, as well as a single PDZ domain (binding to Crb and Baz, Bachmann, Schneider et al. 2001; Hong, Stronach et

al. 2001; Roh, Makarova et al. 2002; Krahn, Buckers et al. 2010), a SH3 domain and two L27 domains (L27N facilitating assembly of the Sdt-PATJ complex and L27C recruiting Lin-7/Veli, Roh, Makarova et al. 2002; Bachmann, Timmer et al. 2004; Li, Karnak et al. 2004; Straight, Pieczynski et al. 2006; Sen, Sun et al. 2015).

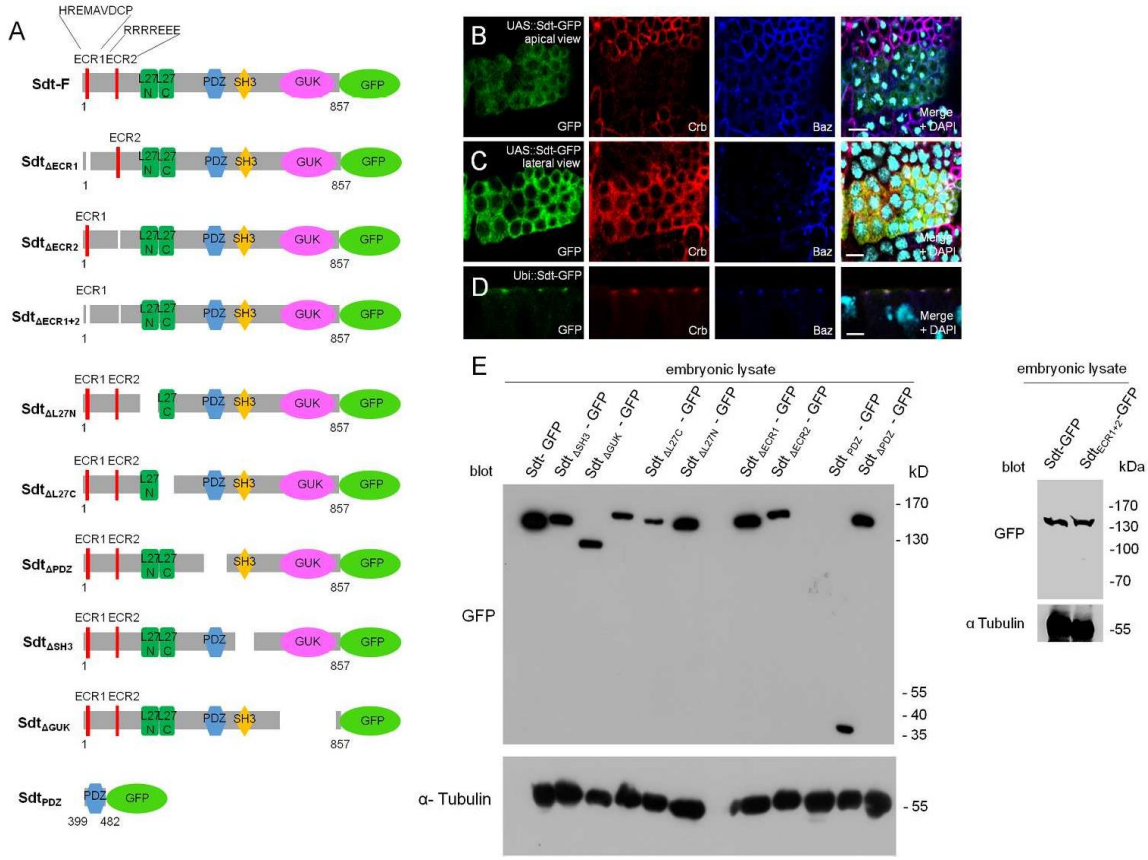

Figure 4.1 Structure-function analysis of *Drosophila* Stardust in epithelial cells. (**A**) Schematic drawing of different Sdt-variants analyzed in this study. The amino acid sequence of ECR1 and ECR2 is indicated in wildtype Sdt. (**B-C**) Overexpression of UAS::Sdt-GFP using en::GAL4 results in cytoplasmic Sdt-GFP localization and depletion of Crb and partially of Baz from the subapical region (the focal plane in **B**) and an accumulation of the proteins in the cytoplasm (more lateral focal plane in **C**). (**D**) Ubi::Sdt-GFP localizes correctly to the apical junctions. (**E**) Western Blotting of embryonic lysates expressing the Sdt variants described in this study. Pictures show embryonic stages 11-12. Scale bars = 5µm.

Furthermore, two highly conserved short amino acid motifs (ECR1 = HREMAVDCP and ECR2 = RRRREEE, Fig. 4.1A) have been identified in mammalian Pals1 to

mediate an interaction with the PDZ domain of PAR-6 (Hurd, Gao et al. 2003; Gao and Macara 2004; Wang, Hurd et al. 2004). Interestingly, the latter interaction is only seen in Sdt-isoforms, which do not exhibit a long spacer between ECR1 and ECR2 due to alternative splicing (formerly annotated as Sdt-B1, Bachmann, Schneider et al. 2001; Berger, Bulgakova et al. 2007; Bulgakova, Kempkens et al. 2008).

Some insights into the *in vivo* relevance of the described protein interactions have already been drawn from structure-function analyses in photoreceptor cells of the developing *Drosophila* eye (Bulgakova, Kempkens et al. 2008), a study in zebrafish using alleles and morpholino-knock-down of Nagie oko, the zebrafish homologue of Sdt (Bit-Avragim, Hellwig et al. 2008), and expression of Pals1-variants in cultured mammalian epithelial cells in a wild-type background (Roh, Makarova et al. 2002).

In order to investigate the relevance of the protein-interaction domains for epithelial polarization in the *Drosophila* epidermis and for the development of the fly, we performed a structure-function analysis of diverse Sdt-variants (as depicted in Fig. 4.1A) in a wild-type background as well as in a *sdt*-mutant background. Even mild overexpression of Sdt (using the UAS/GAL4-system with a weak *armadillo* promoter or *engrailed* promoter at 18°) causes a strong cytoplasmic mislocalization of the overexpressed protein as well as of Crb, polarity defects and embryonic lethality (Fig. 1B and C and data not shown). To avoid these overexpression artifacts, we expressed Sdt from a ubiquitous promoter (*ubiquitin*, *Ubi*), resulting in a physiological localization at subapical region (Fig. 4.1D) and a robust rescue capacity (Fig. 4.4A). We decided to focus on the Sdt-F isoform (formerly annotated as Sdt-B1, Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001; Berger, Bulgakova et al. 2007; Bulgakova, Rentsch et al. 2010) because this isoform has been reported to be expressed throughout embryonic development and to be capable of interacting with PAR-6 as outlined above. We confirmed by Western blotting the expression of a band around 110kDa (predicted size of Sdt-F: 94kDa), which likely corresponds to Sdt-F, from gastrulation onwards (Fig. S4.1B). In addition, a second specific band appeared slightly above, which either corresponds to a newly identified isoform (Sdt-D,

Bulgakova et al. 2010) or represents protein modifications of Sdt-F. Notably, Sdt-B (previously annotated as Sdt-MAGUK1, Bachmann et al. 2001 or Sdt-A, Berger et al. 2007, predicted size 139kDa) is only expressed at very low levels at the end of embryogenesis (Fig. S4.1B, asterisk).

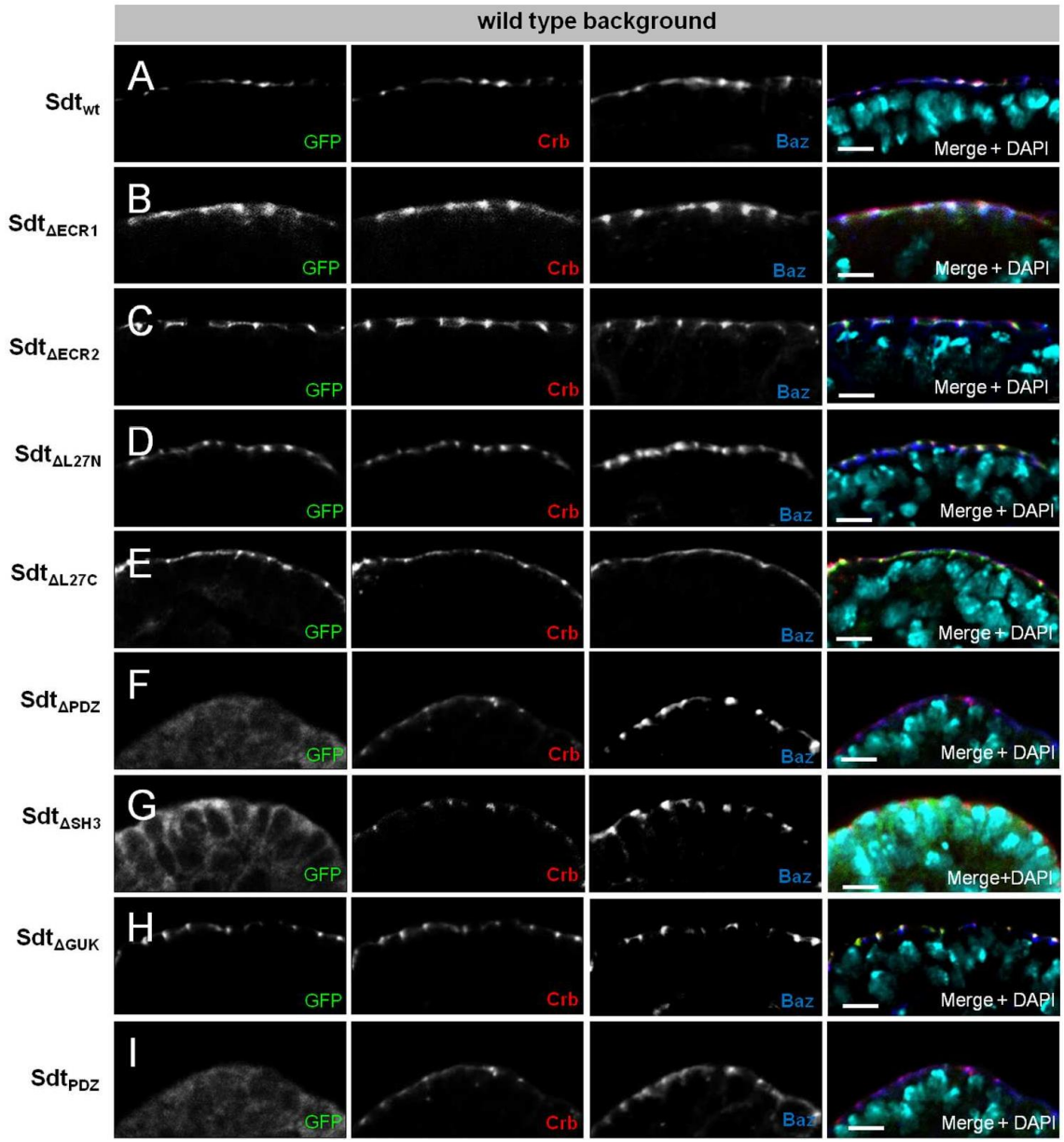

Figure 4.2 Localization of Sdt variants in wild-type epithelial cells. (**A-E, H**) Deletion of the ECR1, ECR2 motif or the L27N, L27C or GUK domain does not impair localization of the mutant protein at the subapical region of wild-type epithelial cells, where it colocalizes with Crb and Baz. (**F**) Sdt protein which lacks the PDZ domain results in a rather cytoplasmic localization of the mutant protein with some residual

staining at the lateral membrane. (**G**) The SH3 domain supports correct targeting to the subapical region and $Sdt_{\Delta SH3}$ mislocalizes – similar to $Sdt_{\Delta PDZ}$ – to the cytoplasm and to the lateral cortex. (**I**) The isolated PDZ domain fused to GFP is not sufficient to localize to the (apical) junctions. All Sdt proteins were expressed with a ubiquitous promoter (Ubi::Sdt-GFP) from two chromosomes carrying the insertion. Pictures show embryonic stages 11-12. Scale bars = 5µm.

Although expression of Sdt-F in a *sdt*-mutant background results in a rescue of around 40% hatched flies and suppression of the apical-basal polarity phenotype in the embryonic epidermis, still approximately 40% of the rescued embryos die at the end of embryogenesis (or fail to hatch as larvae), showing no obvious polarity defects Fig. 4.3B and Fig. 4.4D and M). This might be either due to the artificial promoter, which expresses Sdt in all tissues and might produce dominant negative effects, or due to the lack of further isoforms, which might be essential in a distinct developmental context (Berger, Bulgakova et al. 2007; Bulgakova, Rentsch et al. 2010). However, with respect to the apical-basal polarity of the embryonic epidermis analyses of *sdt*-mutant embryos rescued with Sdt-F show a full rescue capacity and no polarity defects (Fig. 4.3B and data not shown). All Sdt variants were expressed with a C-terminal GFP and can be detected at the correct size in Western Blotting (Fig. 4.1E).

The PDZ and SH3 domain of Sdt cooperate to stabilize the Crb-Sdt complex - First we investigated which domains are necessary to target the protein to the subapical region. In agreement with previous reports, the PDZ domain, which recruits Sdt either to Crb or to Baz at the subapical region (Bachmann, Schneider et al. 2001; Hong, Stronach et al. 2001; Roh, Makarova et al. 2002; Bit-Avragim, Hellwig et al. 2008; Krahn, Buckers et al. 2010), is essential for correct subcellular localization of Sdt. Deletion of the PDZ domain results in a strongly disturbed and mostly cytoplasmic localization of the mutant protein (Fig. 4.2F), whereas wild-type Sdt-GFP correctly localizes to the subapical region (Fig. 4.1D and 4.2A).

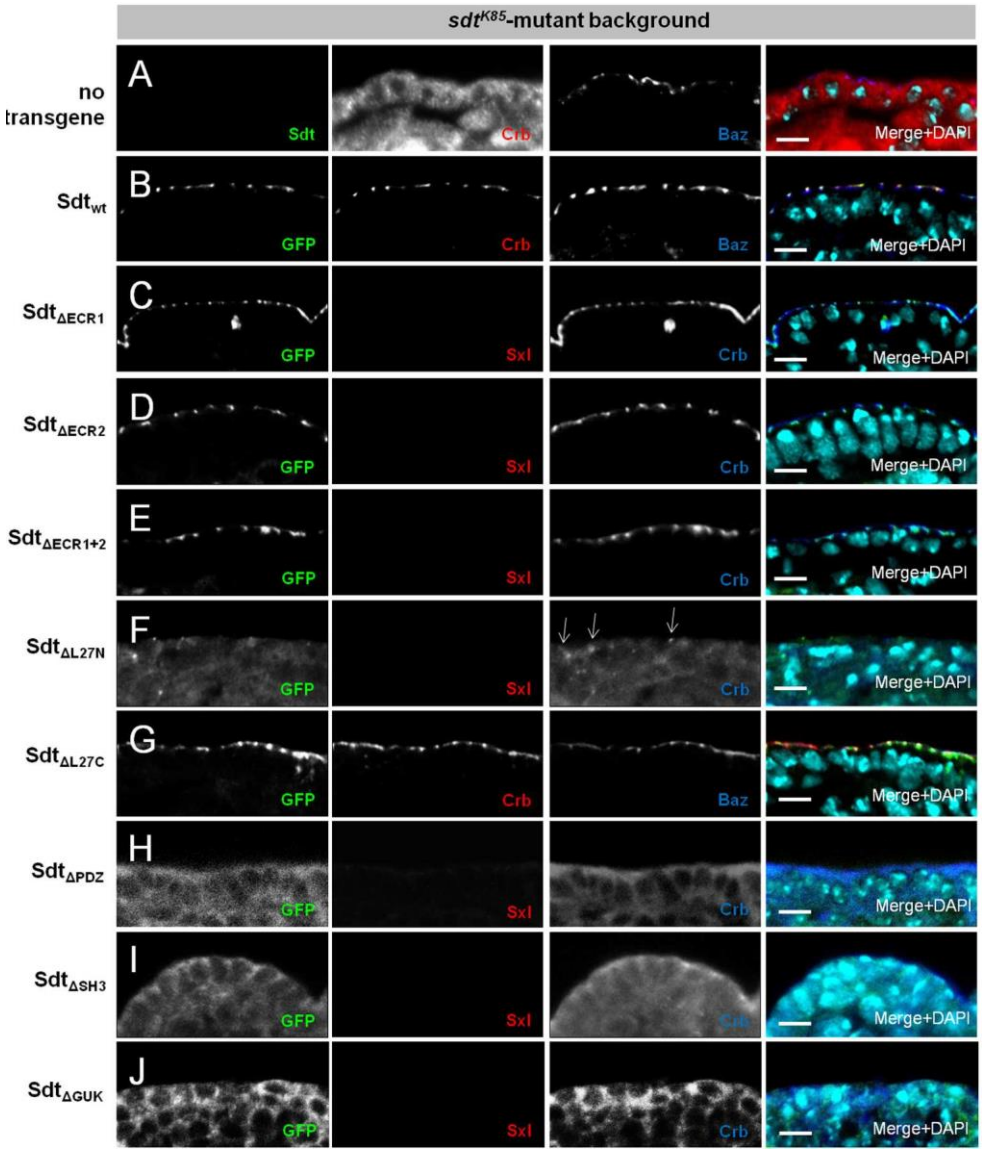

Figure 4.3 Several protein-protein interaction domains of Sdt are essential for epithelial cell polarity and fly development. (**A) (B-E, G**) Expression of wildtype Sdt, $Sdt_{\Delta ECR1}$, $Sdt_{\Delta ECR2}$, $Sdt_{\Delta ECR1+2}$ or $Sdt_{\Delta L27C}$ in *sdt*-mutant cells of the embryonic epidermis rescues apical-basal polarity and Crb localization at the subapical region. (**F, H-J**) Expression of $Sdt_{\Delta L27N}$, $Sdt_{\Delta PDZ}$, $Sdt_{\Delta SH3}$ or $Sdt_{\Delta GUK}$ in *sdt*-mutant embryos results in disturbed apical-basal polarity and mislocalization of Crb. sdt^{K85}-mutant embryos were either derived from homozygous parents (Sdt_{wt}, $Sdt_{\Delta L27C}$) or germ line clones were generated as described in materials and methods and hemizygous mutant embryos were identified using staining against sex lethal. All Sdt proteins were expressed with a ubiquitous promoter (Ubi::Sdt-GFP) from two chromosomes carrying the insertion. Pictures show embryonic stages 11-12. Scale bars = 5µm.

Strikingly, the PDZ domain alone is not sufficient to accomplish cortical recruitment (Fig. 4.2I), although the small PDZ-GFP chimeric protein is stable (Fig. 4.1E). A

recent study suggests that apart from the PDZ domain, the SH3 and GUK domains play a crucial role to support the formation and stability of the Crb-Pals1 complex (Li, Wei et al. 2014). Indeed deletion of the SH3 domain results in a rather cytoplasmic localization of the mutant protein (Fig. 4.2G), although some protein remains at the cell membrane but exhibits a broader localization along the lateral membrane. Although $Sdt_{\Delta PDZ}$ or $Sdt_{\Delta SH3}$ are still capable to bind endogenous PATJ (Fig. 4.5D), PATJ is not displaced from the subapical region by the cytoplasmic Sdt variants (Fig. S4.2B and C). Co-immunoprecipitation experiments of mutant Sdt-proteins with endogenous Crb demonstrate that apart from the PDZ domain, the SH3 domain of Sdt is indeed essential for the association of Sdt with Crb (Fig. 4.5D). Consequently, the expression of $Sdt_{\Delta PDZ}$ or $Sdt_{\Delta SH3}$ does not rescue the cuticle phenotype of sdt^{K85} (Fig. 4.4C, D, J and K) or Crb localization / apical-basal polarity in epithelial cells of the embryonic epidermis (Fig. 4.3H and I). These results are in line with previous studies in zebrafish retinal neural and pigmented epithelia and *Drosophila* photoreceptor cells describing a crucial role of the PDZ domain (Bit-Avragim, Hellwig et al. 2008; Bulgakova, Kempkens et al. 2008) and the SH3 domain (Bulgakova et al 2008) in these apical-basal polarized cell types. In contrast, deletion of the SH3 domain does not affect targeting of Pals1 to the tight junctions (TJ) in cultured mammalian cells (Roh, Makarova et al. 2002).

In contrast, deletion of the GUK domain does not affect localization of the mutant protein in wild-type epithelial cells or its association with Crb (Fig. 4.2H and Fig. 4.5D).

The ECR domains are not essential for apical-basal polarity in the embryonic epidermis - Apart from the canonical Crb-Sdt-PATJ complex, several interactions between the PAR/aPKC and the Crb complex have been suggested: endogenous Sdt and PATJ associate with Baz (but not in a quarternary complex Crb-Sdt-PATJ-Baz), likely by direct binding of Sdt to Baz (Krahn, Buckers et al. 2010; Sen, Sun et al. 2015). aPKC binds to and phosphorylates Crb *in vitro* (Sotillos, Diaz-Meco et al. 2004), although this phosphorylation is not essential for epithelial polarity and fly

development (Huang, Zhou et al. 2008 and unpublished observation). PAR-6 binds directly to the ERLI motif of Crb (Lemmers, Michel et al. 2004; Kempkens, Medina et al. 2006) as well as to the ECR1/ECR2 domains of Pals1 (Hurd, Gao et al. 2003; Gao and Macara 2004; Wang, Hurd et al. 2004) *in vitro* and under overexpression conditions in mammalian cultured cells. An *in vivo* relevance of the Sdt-PAR-6 interaction might be deduced from the mislocalization of PAR-6 in *sdt*-mutant photoreceptor cells which are rescued by Sdt-variant lacking the entire N-terminus including the ECR-motifs and the L27N domain (Bulgakova, Kempkens et al. 2008). We found that $Sdt_{\Delta ECR1}$ and $Sdt_{\Delta ECR2}$ localize correctly at the subapical region in the embryonic epidermis (Fig. 4.2B and C). Furthermore, expression of these transgenes as well as the double mutant protein ($Sdt_{\Delta ECR1+2}$) in a *sdt*-mutant background restores epithelial polarity and Crb localization (Fig. 4.3C-E). However, lethality tests reveal a higher embryonic lethality of *sdt*-mutants expressing either $Sdt_{\Delta ECR1}$ or $Sdt_{\Delta ECR2}$ compared to wildtype Sdt, with an even more increased lethality phenotype for $Sdt_{\Delta ECR1+2}$ (Fig. 4.4A), although immunostainings with polarity markers (Fig. 4.3C-E and data not shown) do not reveal obvious polarity defects. Furthermore, cuticle preparations demonstrate secretion defects in only a minority of embryos (Fig. 4.4E-G, M). Hatched flies of these genotypes appear normal (data not shown). We confirmed that Sdt and PAR-6 can interact *in vitro* and under overexpression conditions in Schneider-2R cells (S2R cells) and that this interaction depends on ECR1 (Fig. 4.5A and B). However, upon immunoprecipitation of endogenous Sdt from wild-type embryos, we were not able to detect substantial amounts of PAR-6 to associate with Sdt whereas Crb, PATJ, Baz and aPKC co-immunoprecipitated with Sdt (Fig. 4.5C). Moreover, mutation of either ECR1 or ECR2 (or both together) does not impair the assembly of the Crb-Sdt complex *in vivo* (Fig. 4.5D).

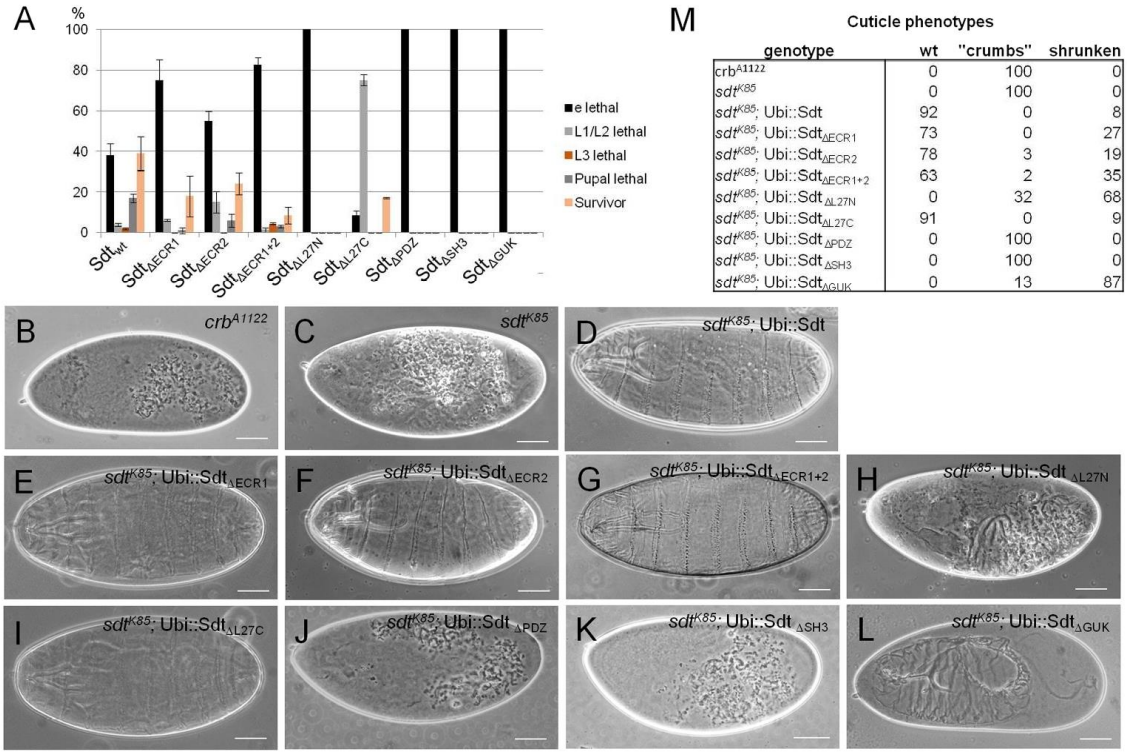

Figure 4.4 Functional analysis of Sdt domains in the development of *Drosophila*. (**A**) Lethality test of different Sdt variants used in this study. For each of the three experiments, 100 homozygous mutant embryos derived from *sdt*^{K85} germ line clones were identified using the FM7-ChFP balancer. (**B-C**) Embryos homozygous mutant for *crb* or *sdt* die during embryonic development and fail to secret more than debris of cuticle ("crumbs" phenotype), whereas expression of Sdt-GFP rescues this defect to a normal cuticle in most embryos (**D** and **M**). The majority of *sdt*-mutant embryos rescued by expressing Sdt$_{\Delta ECR1}$ (**E** and **M**), Sdt$_{\Delta ECR2}$ (**F** and **M**), Sdt$_{\Delta ECR1+2}$ (**G** and **M**) or Sdt$_{\Delta L27C}$ (**I** and **M**) exhibit a regular cuticle. In embryos with Sdt$_{\Delta L27N}$-rescue, one third of the embryos exhibit a "crumbs" phenotype, whereas in two third of the embryos the cuticle is shrunken, shows holes and lacks head structures (**H** and **M**). Embryos with Sdt$_{\Delta PDZ}$-(**J** and **M**) or Sdt$_{\Delta SH3}$-rescue (**K** and **M**) phenocopy the cuticle phenotype of *sdt*. (**L** and **M**) Rescue of *sdt* with Sdt$_{\Delta GUK}$ produces a shrunken cuticle in most cases with holes and defects in head development. 100 cuticles were scored for each genotype. Scale bars = 200µm.

These data suggest that the ECR1 and ECR2 motifs are not essential for a robust binding (which can be detected by co-immunoprecipitation) of Sdt with PAR-6 or a stabilization of the Crb-Sdt-PATJ complex under endogenous conditions and are dispensable for apical-basal polarity but are to some extent crucial for efficient embryonic development.

The L27N domain exhibits functions beyond binding of PATJ - We recently revealed that association of PATJ with both apical polarity complexes (the Crb- and the Baz-complex) is essential for its function (Sen, Sun et al. 2015). The recruitment to these polarity landmarks is facilitated by the L27N domain of Sdt, which heterodimerizes with the L27 domain of PATJ (Li, Karnak et al. 2004). Consequently, deletion of the L27 domain in PATJ abolishes binding to Sdt and localization of the mutant protein at the subapical region (Penalva and Mirouse 2012; Sen, Sun et al. 2015), vice versa, deletion of L27N in Sdt does not affect the protein's localization in wild-type epithelial cells (Fig. 4.2D) but disturbs binding to PATJ (Fig. 4.5D). We and others revealed that PATJ is not crucial for apical-basal polarity or stabilization of the Crb-Sdt complex in the embryonic epidermis (Penalva and Mirouse 2012; Sen, Nagy-Zsver-Vadas et al. 2012; Zhou and Hong 2012) but *PATJ*-mutant flies die during early puparation, showing no obvious metamorphosis, which is – at least partly – due to a decreased Myosin activation (Sen, Nagy-Zsver-Vadas et al. 2012). Surprisingly *sdt*-mutant flies expressing the $Sdt_{\Delta L27N}$ transgene show a complete embryonic lethality (Fig. 4.4A), exhibiting strong polarity and cuticle defects with some residual intact cuticle left, which might be due to some apically localized Crb protein (Fig. 4.3F, arrows and Fig. 4.4H).

A possible explanation for this finding is that L27N facilitates the formation of a stable supramolecular Crb-Sdt(-PATJ) complex by either hetero oligomerization with PATJ or homooligomerization with another Sdt molecule. However the first possibility can be ruled out because loss of PATJ does not affect the stability of Crb-Sdt complex in the embryonic epidermis (Penalva and Mirouse 2012; Sen, Nagy-Zsver-Vadas et al. 2012; Zhou and Hong 2012) and embryos which are homozygous mutant for *PATJ* do neither show polarity defects in the embryonic epidermis nor exhibit a fully penetrant embryonic lethality (Sen, Nagy-Zsver-Vadas et al. 2012). The second possibility is very unlikely because the first homooligomerization of Sdt/Pals1 seems to be biochemically unfavorable (Li, Karnak et al. 2004) and secondly, $Sdt_{\Delta L27N}$ robustly associates with endogenous Crb (Fig.

4.5D). A third explanation would be that unbound PATJ (which does not associate with Sdt anymore) exhibits a dominant negative effect (as overexpression of PATJ does, Sen, Nagy-Zsver-Vadas et al. 2012). However, we did not observe a rescue effect of *sdt/PATJ* double mutants expressing SdtΔL27N, which would support this hypothesis (data not shown).

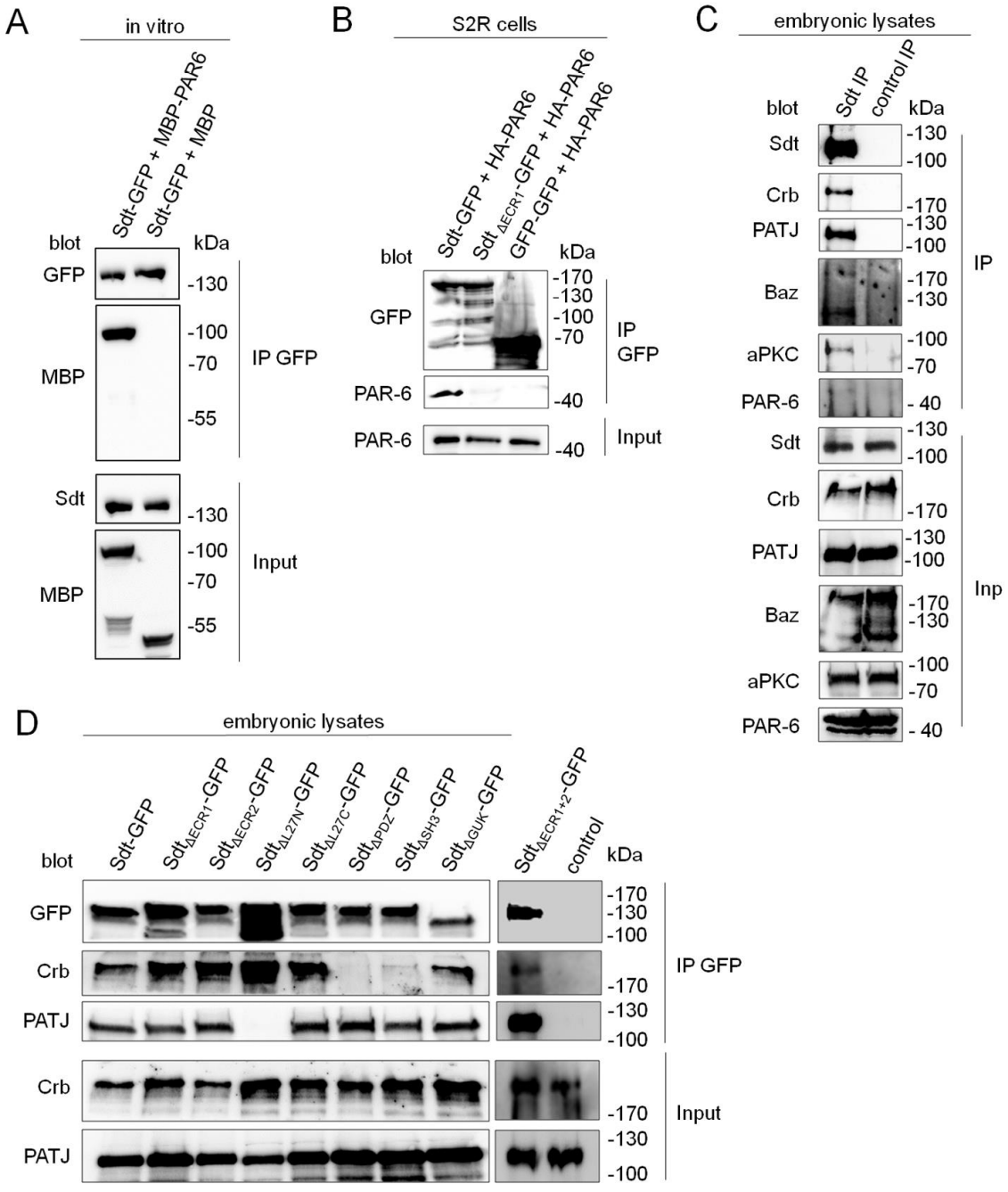

Figure 4.5 Association of Sdt with different polarity proteins. (**A**) Recombinant

MBP-PAR-6 but not MBP alone interacts directly with Sdt-GFP, which was purified from transfected S2R cells. (**B**) Overexpressed HA-PAR-6 co-immunoprecipitates with Sdt-GFP but not with $Sdt_{\Delta ECR1}$ or 2xGFP (negative control). (**C**) Endogenous Crb, PATJ, Baz, and aPKC but not PAR-6 co-immunoprecipitates with endogenous Sdt from embryonic lysates. Control IP = IP with anti-β-galactosidase antibody. (**D**) Sdt-variants were immunoprecipitated using an anti-GFP antibody, and proteins bound to the chimeric proteins were identified by Western Blotting. All Sdt proteins were expressed with a ubiquitous promoter (Ubi::Sdt-GFP) from two chromosomes carrying the insertion.

Thus the L27N domain might accomplish more crucial functions during epithelial polarization of the embryonic epidermis apart from recruiting PATJ.

In contrast to the L27N domain, the L27C domain does not seem to be essential for epithelial polarization and fly development because SdtΔL27C can rescue the polarity phenotypes of sdt^{K85} and produces surviving flies (Fig. 4.3G and Fig. 4.4A and I). This is in line with previous results that identify Lin-7/Veli as an interaction partner of SdtL27C and describe a role for Lin-7/Veli in postsynaptic signal transmission but not for epithelial polarity or fly development (Bachmann, Timmer et al. 2004; Soukup, Pocha et al. 2013). The increased early larval lethality might indeed be due to defects in synapse formation. Although this is in contrast to findings in mammalian cells, which suggest a supportive role of Veli in stabilization of the Crb-Pals1-complex (Straight, Pieczynski et al. 2006), we could not detect a decreased targeting of apical-junctional $Sdt_{\Delta L27C}$ or Crb or a weaker association of mutant Sdt with Crb (Fig. 4.5D).

The GUK domain is not essential for stabilization of the Crb-Sdt-complex but necessary for embryonic development - As mentioned above, deletion of the GUK domain affects neither localization of Sdt to the subapical region in wild-type cells nor its association with Crb (Fig. 4.2H and Fig. 4.5D). However, in a sdt^{K85}-mutant background, expression of $Sdt_{\Delta GUK}$ does not rescue the apical localization of Crb or the embryonic lethality (Fig. 4.4A and 4.3J). Epithelial polarity and embryonic morphology are severely disrupted, which is reflected by a disturbed cuticle secretion:

cuticles from *sdt*^{K85}; Sdt$_{\Delta GUK}$ embryos are shrunken and display big holes in the dorsal cuticle (Fig. 4.4L). This is different from *sdt*^{K85} cuticles which exhibit only crumbles of cuticle and thus phenocopies a *crb* loss of function allele (Fig. 4.4B and C). In contrast to *sdt*^{K85}-mutant embryos (Fig. 4.3A), in which Crb is cytoplasmic/vesicular, *sdt*^{K85}; Sdt$_{\Delta GUK}$ embryos exhibit to some extent a cortical localization, although randomly distributed (Fig. 4.3J). However, this residual cortical localization seems to be sufficient to produce a segmented, although shrunken and irregular cuticle (Fig. 4.4L).

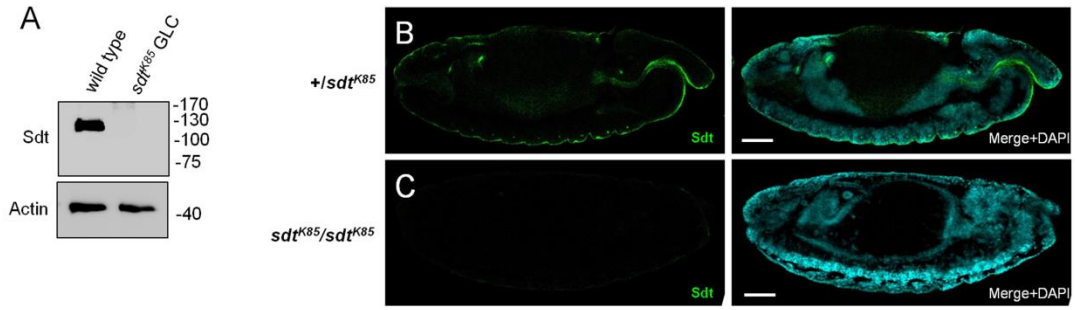

Figure S4.1 Expression of Sdt in embryogenesis. (**A**) Extracts of 0-16h old embryos of either *sdt*^{K85} germ lines clones (mated with males carrying an FM7-ChFP balancer and sorted against ChFP) or wildtype flies were blotted against Sdt and Actin. Both bands seen above the 100kDa marker band appear to be specific. (**B-C**) Heterozygous and zygotic mutant *sdt*^{K85} embryos were stained with anti-Sdt and DAPI. Note that the morphology of *sdt*^{K85} mutant embryos is already disturbed. Scale bars = 50µm.

Therefore we cannot verify a role for the GUK-domain of Sdt in stabilizing the Crb-Sdt complex *in vivo*. Nonetheless, the GUK domain is essential for the function of Sdt during epithelial polarization, which might be independent of its canonical role to stabilize Crb.

Taking together our findings confirms an essential role of the PDZ-SH3 domain tandem in binding and stabilization of Crb *in vivo*. In contrast we did not confirm a function of ECR1 and ECR2 in epithelial polarization *in vivo* – binding of Sdt to PAR-6 does not seem to take place at a substantial level in embryonic epithelia and, as

$Sdt_{\Delta ECR1}$ and $Sdt_{\Delta ECR2}$ mutant embryos do not exhibit polarity phenotypes, it is unlikely that the PAR-6/Sdt interaction contributes to the establishment of apical-basal polarity but might rather have other functions, which are not related to Crb-stabilization. This is in contrast to the function of these domains in photoreceptor cells, where the N-terminus of Sdt-H (originally described as Sdt-B2, an isoform which is exclusively expressed in heads), including ECR1, ECR2, and L27N, is important for the recruitment of PAR-6 in pupae and localization to the stalk membrane in adults (Bulgakova, Kempkens et al. 2008). However, it remains to be clarified whether this phenotype is due to loss of PAR-6 binding (upon deletion of ECR1 and ECR2) or due to impaired binding of Sdt to PATJ (deletion of the L27N domain), or both. Indeed, loss of PATJ in photoreceptor cells results in mislocalization of Crb and PAR-6 (Zhou and Hong 2012).

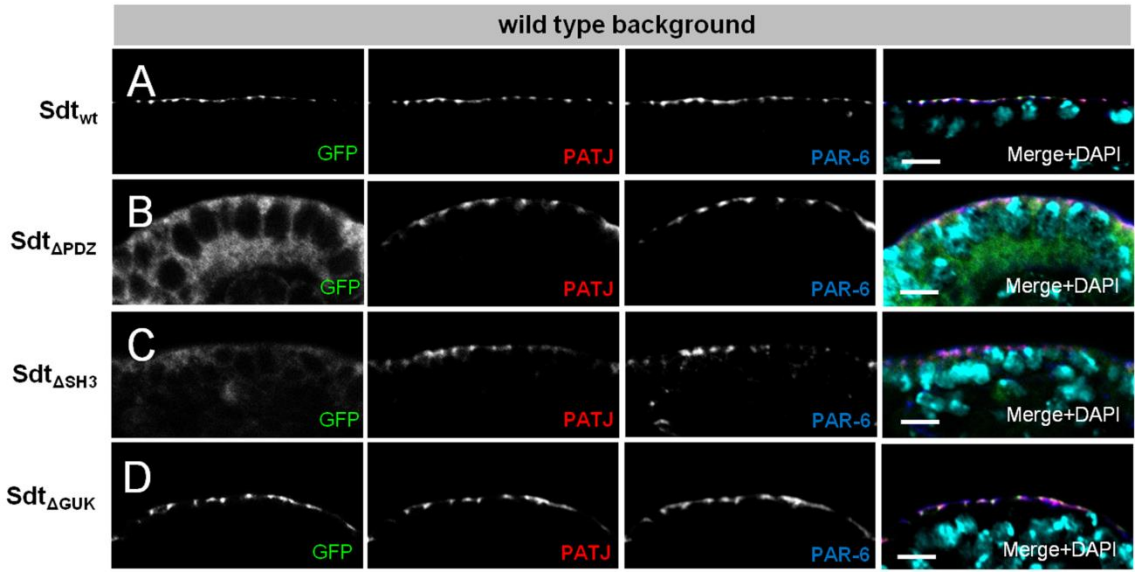

Figure S4.2 Mislocalized Sdt variants do not displace endogenous PATJ or PAR-6. (**A-D**) Sdt-GFP was ubiquitously expressed in wildtype embryos and distribution of GFP, PATJ and PAR-6 was analyzed in stage 11-12 embryos. Scale bars = 5µm.

On the other hand, Sdt isoforms containing a larger stretch of amino acids between ECR1 and ECR2 (e.g. Sdt-B) are not capable of fully targeting PAR-6 in photoreceptor cells. Similar, the ECR1 and L27N domains are essential for retinal

neuron organization and myocard development in zebrafish (Bit-Avragim, Hellwig et al. 2008), whereas ECR1 seems to be dispensable for apical-basal polarity in the retinal pigmented epithelium and the overall fish morphology. This further underlines the importance of the cellular and temporal context for the function of Sdt in polarized tissues, suggesting a crucial role of the Sdt-PAR-6 interaction in neural cells (*Drosophila* photoreceptor cells and zebrafish retinal neurons) but not in other epithelia (*Drosophila* epidermis, retinal pigmented epithelium).

Moreover, our data indicate a new, PATJ-independent function of the L27N domain which is in line with previous reports demonstrating that mutant Nagie oko protein, which cannot bind PATJ fails to rescue body form defects and the neural retinal phenotype of a Nagie oko mutant allele in zebrafish (Bit-Avragim et al. 2008). Deletion of the L27N domain does not affect localization of the mutant protein in the epidermis of wild-type *Drosophila* (which is in agreement with the observation that deletion of PATJ does not affect Sdt localization, Sen et al. 2012), whereas this domain seems to be crucial for TJ targeting of Pals1 in cultured mammalian cells (Roh, Makarova et al. 2002).

Finally, we demonstrate that the GUK domain of Sdt is crucial for the apical-basal polarity of the embryonic epidermis and for embryonic development. Similar, deletion of the GUK domain is indispensable for photoreceptor polarity in the adult *Drosophila* eye (Bulgakova, Kempkens et al. 2008). However, we demonstrate that the GUK domain is neither essential for stabilizing binding to Crumbs as suggested recently (Li, Wei et al. 2014) nor important for the localization of the protein in wild-type epithelial cells. These data hint to an essential, Crb-independent role of the GUK domain during epithelial polarization and embryonic development which was not addressed in previous studies (Roh, Makarova et al. 2002; Bit-Avragim, Hellwig et al. 2008; Bulgakova, Kempkens et al. 2008).

Discussion

The apical-basal polarity of epithelia is controlled by evolutionarily conserved complexes which are determining apical-basolateral domains (Tepass 2012; Thompson 2013). The PAR-aPKC complex defines together with Crb complex the apical region of the cell. The activity and stability of these two complexes are counterbalanced by proteins which localize in the basolateral region, called the basolateral complex. After years of study, it is largely accepted that these complexes are highly dynamic and their components and/or protein modifications are rather diverse in the developmental and cell-specific manner (Hurd, Gao et al. 2003; Nam and Choi 2003; Sotillos, Díaz-Meco et al. 2004; Wang, Hurd et al. 2004; Kempkens, Médina et al. 2006; Krahn, Bückers et al. 2010).

During the development of *Drosophila* embryos, PATJ forms an important protein complex with Crb-Sdt (the canonical Crb complex) and localizes to the apical region when the mature epithelium is established (Sen, Nagy-Zsvér-Vadas et al. 2012). However, at this stage Crb is not expressed, pointing out the question that how the apical targeting of PATJ is mediated. Based on the previous data and the current study, we conclude that in parallel to the canonical Crb-Sdt-PATJ complex, a Baz-Sdt-PATJ complex exists in the *Drosophila* embryonic epidermis. This complex is important for the targeting and the potential functionality of PATJ (Krahn, Bückers et al. 2010; Sen, Sun et al. 2015).

As a core component of PAR-aPKC complex, the PB1 and PDZ domain containing protein PAR-6 plays crucial roles in regulating apical-basal polarity in various epithelia from different organisms (Lin, Edwards et al. 2000; Petronczki and Knoblich 2001; Yamanaka, Horikoshi et al. 2001; Hutterer, Betschinger et al. 2004; Atwood, Chabu et al. 2007; Graybill, Wee et al. 2012). One of these important roles is believed to be dependent on its promiscuous interaction with the Crb complex (Hurd, Gao et al. 2003; Kempkens, Médina et al. 2006). In line with previous studies, PAR-6 regulates

the Crb complex stability and correct apical localization (Kempkens, Médina et al. 2006). Moreover, we have found that PAR-6 inhibits the proteasomal receptor Rpn13 dependent Sdt degradation. Thus, a link is established between protein quality control and cell polarity regulation.

1. PATJ as a component in two distinct apical complexes

As the third member of Crb complex, PATJ directly associates with Sdt in the heterodimerized manner and in turn, PATJ binds to Crb and forms Crb-Sdt-PATJ complex (Bulgakova and Knust 2009). The well-defined Crb complex localizes to the tight junctions in vertebrates and to the corresponding subapical region in *Drosophila* (Roh, Makarova et al. 2002; Bulgakova and Knust 2009).

Upon the maturation of apical-basal axis in late cellularization/early gastrulation during *Drosophila* embryogenesis, PATJ-Sdt complex localizes to the apical region while Crb is absent, indicating the existence of alternative protein complex which is independent on Crb. Indeed, the previous study shows that Sdt is initially targeted to the apical membrane by the formation of Baz-Sdt complex. This is achieved by the direct interaction between PDZ domain of Sdt and aPKC binding domain of Baz (Krahn, Bückers et al. 2010). In line with this study, the localization of Sdt and PATJ was abolished in a maternal and zygotic (germline clones, GLC) *baz* mutant embryo at stage seven before the expression of Crb. This phenotype could be explained by the stage-dependent biochemistry evidences (Co- immunoprecipitations). Specifically, PATJ and Sdt could be co-precipitated with Baz in the embryos from stage one to stage nine (middle of germ band elongation). These data strongly indicate that before the onset of Crb expression, PATJ forms a complex with Baz-Sdt, which is crucial for its early apical targeting in *Drosophila* embryonic epidermis. Interestingly, this Baz-Sdt-PATJ complex seems to be preserved during the further development since they co-precipitated from the later stages of embryos. Notably, the targeting of PATJ is highly dependent on Sdt as *sdt* mutants completely abolish the apical localization of PATJ and Sdt binding deficient PATJ variant does not localize correctly. Collectively,

these findings point out the fact that in parallel to the canonical Crb complex, PATJ also assembles with Baz-Sdt. These two complexes co-exist during *Drosophila* embryonic development.

Apart from directing the apical localization of PATJ, the functions of Baz-Sdt-PATJ complex are largely unknown. Recent studies show that PATJ is not essential for Crb-Sdt complex formation during early embryonic development, but rather functions in regulating myosin activity via directly binding to myosin binding subunit (MBS) of myosin phosphatase, therefore supporting adherens junction (AJ) stability (Pénalva and Mirouse 2012; Sen, Nagy-Zsvér-Vadas et al. 2012; Zhou and Hong 2012). Indeed, MBS associated with both complexes, indicating the possibility that PATJ regulates the myosin activity in two distinct complexes. Interestingly, Baz interacts with DE-Cadherin and it is believed to be the alternative mechanism to stabilize AJ in *Drosophila* (Bulgakova, Grigoriev et al. 2013). Thus, the AJ-stabilization function of Baz might be achieved by recruiting PATJ to the complex, thereby indirectly regulating myosin activity. So, association of PATJ with two complexes might control cytoskeleton assembly and contractility in redundancy.

Besides, one thing to note is that in contrast to the embryonic epidermis, the localization of PATJ and Sdt is absolutely independent on Baz in *Drosophila* follicular epithelium since they had normal apical localizations in the *baz* mutant cells. In line with previous findings, the interaction with Crb seems to be more important for Sdt and PATJ's apical localization in follicular cells (Shahab, Tiwari et al. 2015; Sen, Sun et al. 2015). These observations emphasize that different epithelia (at least embryonic epidermis and follicular epithelium) have different polarity machineries.

There are two homologous of *Drosophila* PATJ which are encoded in the mammalian system: PATJ/INADL and Mupp1. These two proteins share very high similarities in both structures and functions (Hamazaki, Itoh et al. 2002; Adachi, Hamazaki et al. 2009). However, PATJ/INADL seems to play more important role in tight junction modulation (Adachi, Hamazaki et al. 2009). Recently, we managed to generate the PATJ/INADL conditional knock-out mice. Surprisingly, the homozygous knockout

mice are viable and fertile without showing any obvious defects regarding polarity (data not shown in this thesis). These data point out the possible redundant and compensated effects between PATJ/INADL and Mupp1. This idea is further supported by the evidence that Mupp1 homozygous knockout mice also appear normal (personal communications with Jackson Laboratory). Thus, it seems necessary to generate the double knock-out strains to investigate the detailed role of PATJ and/or Mupp1 in mammals.

2. PAR-6 regulates the stability of the Crb complex

PAR-6 shows the promiscuous interacting pattern with Crb-Sdt/Pals1-PATJ *in vitr*o (Hurd, Gao et al. 2003; Gao and Macara 2004; Lemmers, Michel et al. 2004; Penkert, DiVittorio et al. 2004; Wang, Hurd et al. 2004; Kempkens, Médina et al. 2006; Nam and Choi 2003). However, all these interactions were validated with the bacterially purified proteins or in over-expression systems using cultured cells. In contrast, our study shows that PAR-6 could not co-precipitate at detectable levels with any components of the Crb complex under the endogenous conditions, indicating that there are no substantial complexes between PAR-6 and Crb-Sdt-PATJ in the *Drosophila* embryonic epithelia. These data also highlight the significant differences between *in vitro* and *in vivo* systems.

The complete Sdt depletion in the *PAR-6* mutant epithelial cells is one of the main findings of this study. Further investigations suggested that the stabilization of Sdt expression by PAR-6 was regulated on a post-transcriptional manner since the *sdt* mRNA level did not change in the *PAR-6* knockout embryos comparing to wildtype ones. The ubiquitin receptor Rpn13 was shown to interact with the PDZ domain of PAR-6 in a yest-2-hybrid screening which was performed in worms (Lenfant, Polanowska et al. 2010). In our current research, this interaction could be confirmed in *Drosophila.* Interestingly, the Rpn13 could also directly interact with Sdt, which was demonstrated by the Co-immunoprecipitations and GST pull-down assays. As Rpn13 is a component of the 26S proteasome, these data point to the possibility that

the Sdt degradation in *PAR-6* mutant cells depends on the proteasomal system. Indeed, a particular Sdt isoform can be targeted by the E3 ligase Neuralized (Perez-Mockus, Roca et al. 2017). Neuralized directly binds Sdt-B isoform and enhance its degradation. The polarity defective phenotype caused by Neuralized deletion can be rescued by overexpressing a Neuralized binding deficient Sdt isoform. However, Sdt has many splicing isoforms and only the one which contain a long stretch between two evolutionarily conserved regions (ECRs) can be targeted by Neuralized. These data indicate that another E3 ligase is involved to complete the degradation of Sdt in *PAR-6* knockout *Drosophila* epithelia.

Crb complex assembly and stability highly depend on the interaction with its intracellular adaptors (Bulgakova and Knust 2009). The PDZ domain of Sdt interacts with the PDZ binding motif (PBM) of Crb and this interaction ensures the apical localization of Crb. Interestingly, the Crb PBM also interacts with α-adaptin, a component of the cargo internalization complex AP-2 (Lin, Currinn et al. 2015). After binding α-adaptin, the endocytosis of Crb is enhanced and as a result, Crb protein level is decreased. In the current study, Crb got mislocalized and displayed to some extend a reduced protein level in the *PAR-6* mutant epithelial cells. As Sdt is degraded completely in absent of PAR-6 and loss-of-Sdt has been reported to induce Crb mislocalization, it is reasonable to propose that α-adaptin might bind more to Crb PBM in absent of Sdt in the PAR-6 knoctout cells, therefore mislocalizing and destabilizing Crb. On the other hand, Crb stability is additionally promoted by its FERM domain binding partners, such as moesin and expanded (further binding to apically localized β-H-spectrin networks) (Médina, Williams et al. 2002; Pellikka, Tanentzapf et al. 2002; Fletcher, Elbediwy et al. 2015). Thus, it might be interesting to elucidate these mechanisms in future studies.

aPKC is a well-known serine/threonine kinase containing a kinase domain and a PB1 domain (Tepass 2012). One of the well-established roles of PAR-6 is to active the kinase activity of aPKC (Yamanaka, Horikoshi et al. 2001; Graybill, Wee et al. 2012). Consequently, aPKC kinase activity was dramatically reduced in the *PAR-6*

knock-down cells, while the aPKC protein level did not show significant changes. This observation led to the Sdt-rescue experiment with over-expressing a constitutively active variant of aPKC in the *PAR-6* mutant cells. Surprisingly, Sdt stability and localization was not restored. Moreover, Sdt and PAR-6 were correctly localized in the aPKC kinase-dead (loss-of-kinase activity variant of aPKC) epithelial cells (Kim, Gailite et al. 2009). These data indicate a kinase-independent function of aPKC in polarity regulation. Consistently, the PB1 domain of aPKC has several important binding partners (including PAR-6) such as MEK and p62 which play important roles in cell growth and autophagy (Yoshinaga, Kohjima et al. 2003; Christian, Krause et al. 2014). Binding of different partners to PB1 domain is crucial to locate and specify the functions of aPKC in different signal cascades (Moscat and Diaz-Meco 2000). This functional specifying process *per se* is independent of the kinase activity of aPKC.

Rpn13 initially named adhesion regulating molecule 1 (Adrm1) is characterized as the intrinsic ubiquitin protein receptor (Hamazaki, Iemura et al. 2006; Jørgensen, Lauridsen et al. 2006; Qiu, Ouyang et al. 2006). We demonstrated a direct interaction between Sdt and Rpn13 *in vitro*. However, Sdt did not interact with Rpn13 under the endogenous conditions when PAR-6 is expressed, suggesting that PAR-6 inhibits Rpn13 binding to Sdt *in vivo*. Furthermore, Rpn13 knockdown/ knockout could rescue Sdt at the cell-cell contacts in *PAR-6* mutant epithelial cells. These data indicate the PAR-6-Rpn13 interaction is functional and crucial to stabilize Sdt in order to regulate epithelial cell polarity. Interestingly, neither Rpn13 overexpression nor knockout impaired the epithelial polarity, suggesting Rpn13 itself is not important in polarity regulation.

Apart from the transcription factor p63, liver kinase B1 (LKB1) and inducible nitric oxide synthase (iNOS), Sdt is another direct target of Rpn13 (Huang and Ratovitski 2010; Huang and Ratovitski 2010; Mazumdar, Gorgun et al. 2010). These findings raise the question whether Rpn13 regulates a certain subgroup of proteins (e.g. cell polarity/junction-associated proteins). Additionally, Rpn13 is found to be

over-expressed in multiple cancer specimens (Anchoori, Karanam et al. 2013; Jang, Park et al. 2014). Knockdown or inhibition of Rpn13 leads to decreased cell migration, cell proliferation and tumorigenicity in cells deriving from different cancers (Anchoori, Karanam et al. 2013; Song, Das et al. 2014; Zheng, Guo et al. 2015; Song, Ray et al. 2016). Therefore, Rpn13 is proposed as a novel target for cancer therapy. Our findings provide another mechanism for Rpn13 targeted therapy which involves epithelial polarity determinants modulation.

The Hippo pathway is highly conserved from *Drosophila* to human and plays a central role in controlling cell proliferation, apoptosis and stemness (Yu, Zhao et al. 2015). Many polarity proteins, including Crb and Sdt/Pals1 have been described to function as the positive regulators of the Hippo pathway, (Ling, Zheng et al. 2010; Robinson, Huang et al. 2010; Varelas, Samavarchi-Tehrani et al. 2010; Zhao, Tumaneng et al. 2011). In *PAR-6* mutant follicular cells, it was quite often to observe the hyperproliferation, indicating the uncontrolled cell growth (Ogawa, Matsuzaki et al. 2009). As the loss of PAR-6 results in the loss of Sdt and consequently the mislocalization of Crb, it is interesting to investigate whether the Hippo pathway is impaired in *PAR-6* mutant cells and through which protein(s) PAR-6 affects this signaling. Since compromised expression of Rpn13 is able to rescue Sdt, it is also of great interest to test whether Rpn13 is involved in Hippo pathway regulation in future studies.

Overall, in our study, we revealed a new protein complex Baz-Sdt-PATJ existing in *Drosophila* embryonic epidermis and the detailed mechanism of PAR-6 in modulating the Crb complex stability and functionality. These results shed new light on the importance of dynamic interactions between the PAR-aPKC complex and the Crb complex in regulating the epithelial polarity in *Drosophila*.

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