# The role of PATJ in the development of *Drosophila melanogaster*

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## Summary

In the due course of the formation of apical-basal polarity the transmembrane protein Crumbs (Crb) and its intracellular adaptor protein Pals1 (Protein associated with Lin seven 1, Stardust, Sdt in *Drosophila*) have been found to play key roles in the establishment and maintenance of cell polarity in various types of tissues. Research in *Drosophila* revealed that PATJ (Pals1 associated tight junction protein) which have been reported to be a part of the trimeric complex with Crb-Sdt localizes at the apical cell-cell contacts and plays roles in the formation of the tight junction and cell migration in mammalian cells. However it is not yet fully understood how PATJ has been localized to the apical cell junctions and its role in the regulation and maintenance of cell polarity.

In this vivid study in elucidating functional significance of PATJ, a systematic structural-functional analysis have been carried out with deletion constructs tagged with green fluorescent protein (GFP) in transgenic flies to elucidate the roles of each conserved domain of the protein. In our study we found that the N-terminally located L27 domain along with a redundancy of the PDZ domains is required for proper functionality of the protein. Further we also found that PATJ attaches to both Baz-Sdt and Crb-Sdt complexes for its proper functionality. On our way to decipher how PATJ shuffles between these two complexes, we reveal the role of PAR6 in stabilizing Crb-Sdt complex via selective inhibition of Sdt degradation by ubiquitin mediated proteosomal pathway.

Additionally we have found that PATJ is not per se crucial for the establishment or maintenance of apical-basal polarity, but rather regulates Myosin dynamics. PATJ directly binds to the Myosin Binding Subunit of Myosin Phosphatase and decreases Myosin dephosphorylation, resulting in activated Myosin dynamics. Thereby PATJ supports the stability of the *Zonula Adherens*. Notably, weakening of Adherens Junction (AJ) in a *PATJ*-mutant epithelium leads first to a loss of Myosin from the AJ, subsequently to a disassembly of the AJ and finally to a loss of apical-basal polarity and disruption of the tissue.

# 2. Introduction

## 2.1. Cell Polarity

Cell polarity mainly arises from the asymmetric division of cells in respect of cell shape, protein distributions and cell functions in different tissues. Cell polarity spans its evolutionary diversity from single cell to multi-cellular organisms. It has been found to function in important biological aspects like the establishment of cell barriers, directed growth, migration of cells and so forth.

To date various forms of cell polarity have been described, such as planar cell polarity, anterio-posterior polarity, apical-basal polarity (Fig.1) and radial cell polarity. Extensive research in the past has shed light on many polarity landmarks which play an active role in the establishment of apical-basal polarity. Fortunately most of the cues are conserved to a greater extent throughout evolution in various organisms from invertebrates like the nematode *Caenorhabditis elegans* or the fruitfly *Drosophila*) to vertebrates (and mammals in particular) (Nelson, 2003).

Riding on the success of developmental biology which opens up a scope to study model organism like *Drosophila melanogaster*, it has been possible to study cell polarity with versatile tools enabling the opportunity to study 'in vivo' various mechanisms, candidate genes, proteins etc. *Drosophila* also provides the possibility to study several cell types which are polarized and serves as a perfect model for investigation.

Several cell types of *Drosophila melanogaster* have been established as model systems for *in vivo* studies on different aspects of cell polarity:

1. The oocyte: Unlike the *C. elegans* oocyte, which lacks polarity before fertilization the *Drosophila* oocyte is a highly polarized cell that contains a large number of localized messenger RNAs and proteins along an anterior-posterior (and dorsal-ventral) polarity

axis. The oocyte is surrounded by the mesodermal derived follicular cell epithelium. Unlike other epithelial cells the apical domain of these follicle cells is not directed towards the lumen or the outside surface; instead it forms cell contacts with the germline cells (oocyte and nurse cells).

2. The ectodermal epithelia: Ectodermal epithelia eg. the epidermis, fore- and hindgut and tracheal system emerge from primary epithelia which originate directly (without any non-epithelial intermediates) from the blastoderm.
3. The neural stem cells (neuroblasts): *Drosophila* neuroblasts offer a nice model for studying asymmetric cell division. Neural development which starts during stage 9 of embryogenesis also provides a distinct model for studying apical-basal polarity.

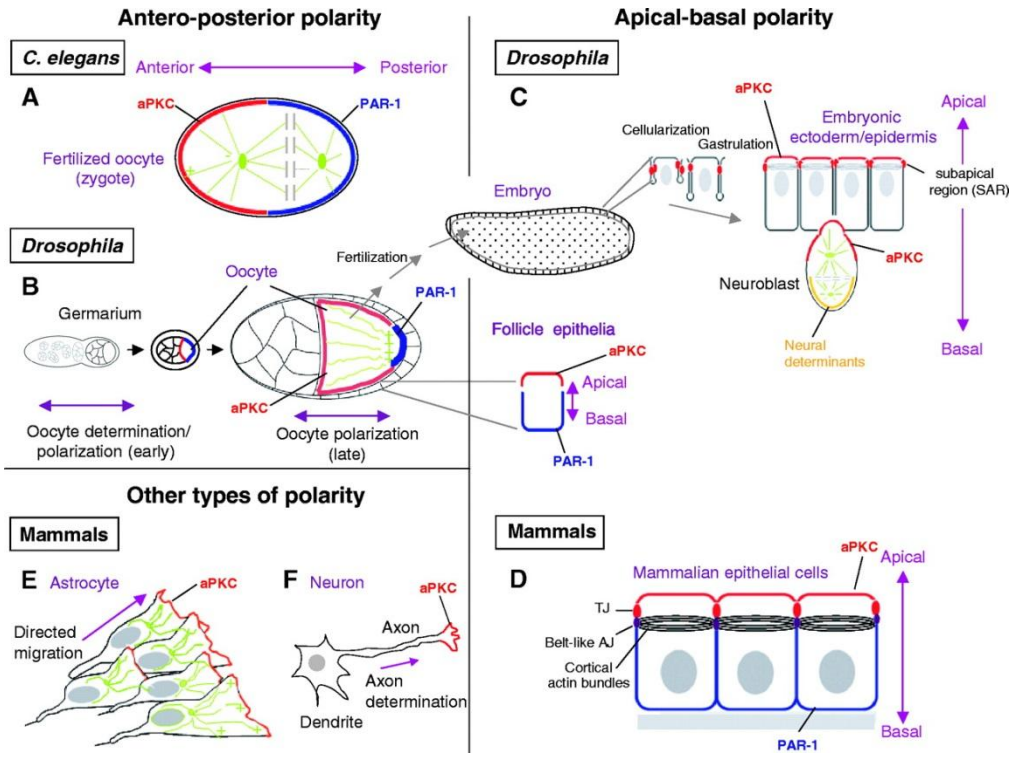


**Figure 1: Various polarized cell types used as a model in *Drosophila melanogaster*** (edited from Suzuki and Ohno 2006)

## 2.2 Epithelial cell polarity in vertebrates and *Drosophila*

The ectodermal epidermis serves as a good model for studying fundamental mechanisms of apical-basal polarity. The first epithelia to form in the *Drosophila* embryo is the blastoderm which develops from a syncytium by multiple invaginations of the plasma membrane causing the formation of the cleavage furrows, a process known as cellularization. With an increase in the surface area and orderly segregation of around 5000 nuclei, establishment of cell polarity takes place concomitantly with the growth of the polarized plasma membrane (Lecuit. et al., 2002). Previous studies on epithelia of various species have revealed many highly conserved genes which are responsible for cell polarity (Knust and Bossinger, 2002).

Apical-basal polarity has been mainly formed by mutual segregation of certain proteins and lipids distributed between certain distinct domains, an apical membrane domain, lateral cell contacts and a basal zone. Often the last two domains are annotated as basolateral domain. Cell-cell and cell-matrix interactions (Wang et al., 1990; O'Brien et al., 2002) and in particular the assembly of apical and basolateral junctional complexes are prerequisites for the proper development of cell polarity: First epithelial cells have an adhesive belt encircling the cell just below the apical domain known as *zonula adherens* (ZA).

In *Drosophila* and vertebrate epithelial cells the transmembrane protein E-Cadherin (and other proteins belonging to the same family) binds directly to β-catenin which in turn recruits α-catenin which forms the pre-requisite for the linkage to the actin cytoskeleton, partly directly and partly via actin binding proteins like vinculin or α-actinin (Nelson, 2008; Perez-Moreno et al., 2003). However further studies have shown that linkage of actin cytoskeleton to the cadherin-catenin complex is more complicated than a simple interaction of the proteins (Weis et al., 2006).

Second the boundary between the apical and lateral domains is marked by the tight junctions (TJ), which contain a number of homophilic adhesion molecules, such as Occludin, Junctional

Adhesion Molecules (JAMs), and the Claudins, which create the paracellular barrier and an intramembranous diffusion barrier between apical and basolateral transmembrane proteins (Johnston et al., 2010) (Fig.2). Apart from the junctional proteins some other transmembrane and cytoplasmic proteins also accumulate near the TJ, namely the PAR/aPKC complex (PAR-3, aPKC, PAR-6) and the Crumbs complex Crumbs (Crb) / PALS1 (protein associated with Lin7) / PATJ (PALS1-associated TJ protein). These apical protein complexes are mutually excluded and controlled by a basolateral Discs Large (Dlg) / Scribble (Scrb) / Lethal (2) giant larvae (Lgl) complex.

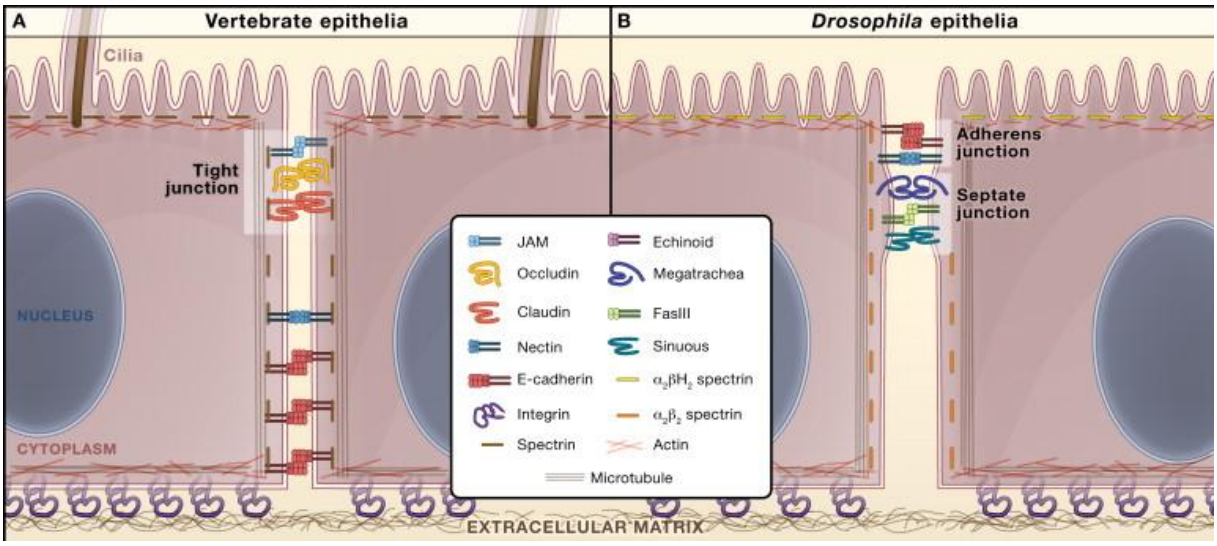


**Figure 2: Intercellular Junctions in Epithelial Cells of *Drosophila and* vertebrates** (from Daniel St Johnston and Julie Ahringer 2010)

In *Drosophila* epithelia components of the AJ are highly conserved with respect to vertebrate system but differ in the arrangement of lateral junctions. As *Drosophila* epithelia do not express Occludins they do not form real TJ, instead they develop a distinct region apical to the ZA, known as sub-apical region (SAR), that localize at a region homologues to the TJ in vertebrate cells (Knust and Bossinger, 2002). The mentioned PAR complex is localized to the ZA and SAR while the Crb complex is located slightly apical to the PAR complex in the SAR. The basolateral proteins like Dlg, Scrib and Lgl are on the other hand localized to a special junction called Septate Junction (SJ). SJs are specific to the invertebrate system but

they do posses analogy to the vertebrate TJs as many TJ specific protein homologues like Claudins [Sinuous (Sinu), Megatrachea (Mega) and Kune-kune (Kune)], the Na+/K+-ATPase, Nrg (Neuroglian), Neurexin-IV (Nrx-IV), Contactin (Cont), Lachesin (Lac), Gliotactin (Gli) and two intracellular components,Coracle (Cora) and Varicose (Vari) are localized to SJ. TJ and SJ also share similar functions in restriction of paracellular molecular transport across epithelial sheets and helping to polarize plasma membrane and the underlying cytoskeletal elements along apical-basal axis (Tepass et al., 2001; Furuse and Tsukita, 2006; Cereijido et al., 2008; Miyoshi and Takai, 2008) (Fig.3).

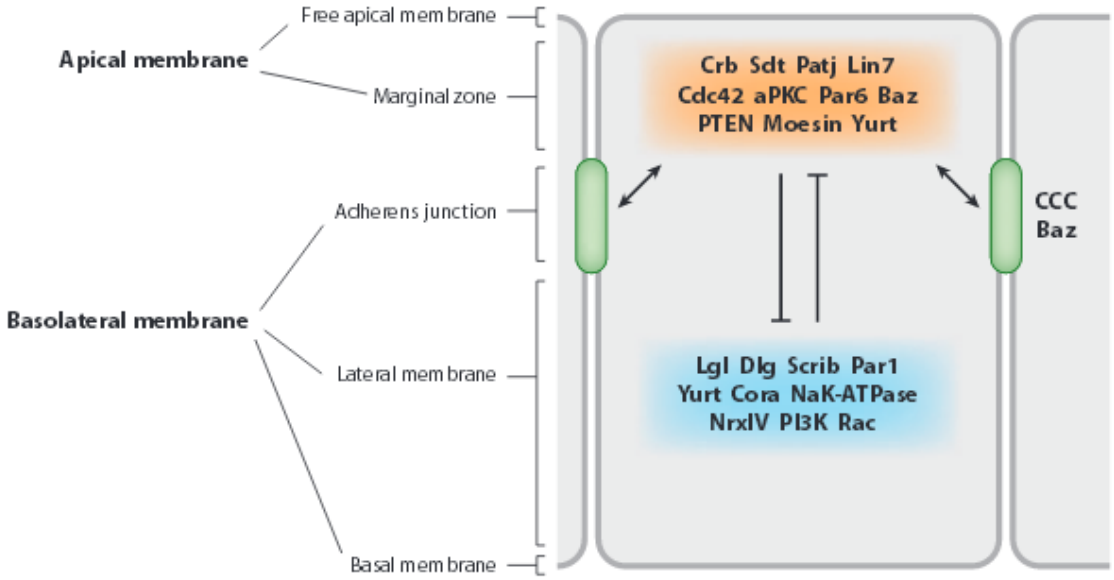


**Figure 3. Localization of protein complexes in the *Drosophila* epithelium** (from Tepass 2012)

## 2.3 The Crumbs complex

The Crumbs complex is comprised of four core proteins: Crb, its intracellular adaptor protein Pals1, PATJ and Lin7 (Fig.4). Crb and Sdt are highly conserved from fly to men and are known to play key roles in the maintenance of apical-basal polarity and integrity in *Drosophila* epithelia and photoreceptor cells as well as in vertebrate epithelia and polarized

cells of the retina (Tepass and Knust, 1993; Tepass et al., 1990; Bulgakova and Knust 2009). The formation of the Crb complex is achieved via physical interaction of the PDZ (**P**sd95, **D**isc large, **Z**O-1) domain of Sdt and the C-terminal ERLI motif of Crb. (Bachmann et al., 2001; Hong et al., 2001). The two L27 domains of Sdt bind to the L27 domains of PATJ and Lin-7 (Bachmann et al., 2004; Bulgakova et al., 2008; Roh et al., 2002). This complex interestingly has an asymmetric localization in the apical cortex in the SAR, just above the ZA, irrespective of species or cell type (Berger et al., 2007; Johnson et al., 2002; Pellikka et al., 2002; Richard et al., 2006a; Tepass, 1996).

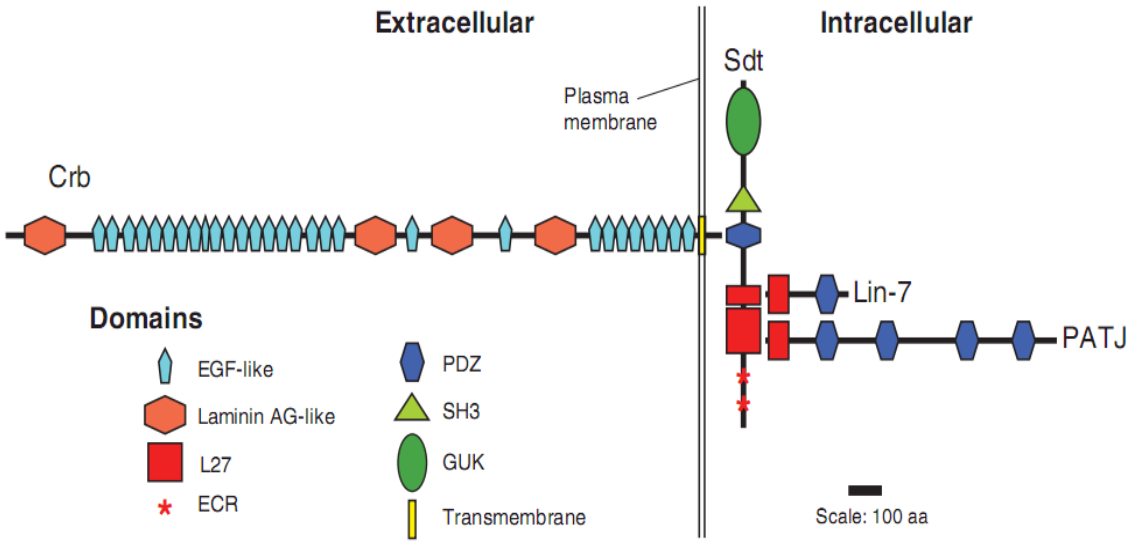


**Figure 4. Schematic diagram of the core proteins of the *Drosophila* Crumbs complex** (from Bulgakova and Knust 2009)

## 2.4 PATJ

The *PATJ* gene has been first identified by Bhaat et al. in a yeast two-hybrid screen for binding partner of Nrx-IV, a component of the SJs in the *Drosophila* embryo. They mistakenly thought to have a PATJ mutant and annotated it as *disc lost* (Dlt) (Bhaat et al,. 1999). Later on it has become evident that Dlt is a neighbouring gene which is unrelated to

PATJ (Pielage et al., 2003). *Drosophila* PATJ contains four PDZ domains and a single L27 (Lin-2, Lin-7) domain at the N-terminus (Pielage et al., 2003) (Fig.5). Studies have reported that PATJ forms the third member of the Crumbs complex by binding to the N-terminal L27 domain of Sdt via its own L27 domain in *Drosophila* embryos (Klebes and Knust, 2000; Roh et al., 2002b) and also in adult flies (Pellikka et al., 2002). In contrast, its mammalian homologue consists of 10 PDZ domains. Another multiple PDZ domain containing protein MUPP1 (13 PDZ domains) is also referred to have close similarity with mammalian PATJ with partly overlapping functions of regulating TJs (Adachi et al., 2009), including binding to Claudins and JAM (Hamazaki et al. 2002, Poliak et al. 2002) (Fig.5).

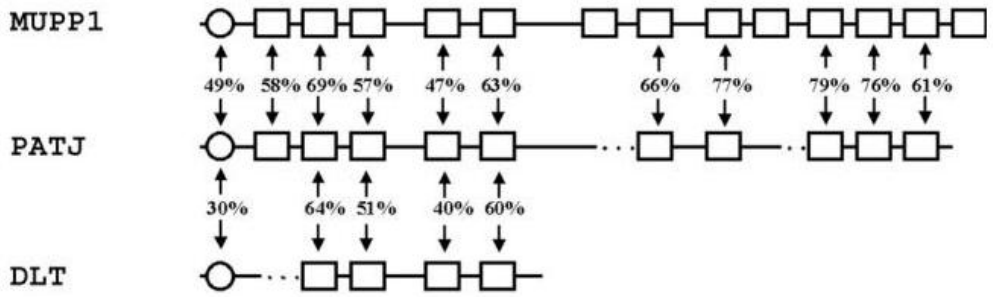


**Figure 5. Alignment of different PATJ homologues in vertebrate and invertebrate system** (edited from Roh et al., 2002)

Of mammalian PATJ's ten PDZ domains, binding partners have been identified for only two. PATJ interacts with ZO-3 via its sixth PDZ domain and with Claudin-1 via its eighth PDZ domain. (Lemmers et al. 2002, Roh et al. 2002a,b). PATJ also have been reported to play a role in development of mammalian cell polarity in MDCKII (Madine-Darby canine kidney) cells (Shin et al., 2005). Suppression of PATJ expression in Caco- 2 (human epithelial colorectal adenocarcinoma) cells resulted in decreased stability of the CRB3 complex and localization of CRB3 to the intracellular compartment (Michel et al. 2005). Furthermore, PATJ is required for the formation of TJ (Latorre et al. 2005). Similar effects were observed overexpressing a dominant-negative version of PATJ in MDCK cells (Hurd et al., 2003). Recent research sheds light on the role of PATJ in apical constriction of epithelial cells via AJ

associated Acto-Myosin belt by modulating direct or indirect recruitment of small GTPase RhoGEFp114 to the apical junction (Nakajima and Tanoue, 2011). In turn Lulu2 (the mammalian homologue of *Drosophila* Yurt) interacts with and activates RhoGEFp114 in regulation of the circumferential Acto-Myosin belt. RNAi knockdown of either PATJ or Lulu2 results in loss of the Acto-Myosin belt and consequently apical constriction (Nakajima and Tanoue, 2012). It has been also shown that PATJ plays significant role in cell migration by wound healing assays (Shin et al., 2007). This observation was supported by the findings of PATJ acting as a scaffold for Angiomotin and the RhoGEF Syx in migrating endothelial cells (Ernkvist et al., 2009).

In *Drosophila* the role of PATJ has been obscurely described in various studies. In follicular epithelium PATJ forms a complex with Crb and have been postulated to regulate the formation of follicle cell epithelium by stabilizing Crb in this cell type (Tanentzapf et al., 2000). In adult *Drosophila* eyes PATJ has been shown to be necessary to stabilize the Crumbs complex at the stalk membrane of photoreceptor cells (Richard et al., 2006). PATJ also have been shown to affect planar cell polarity (PCP) through interaction of one of the key players Frizzled (Djiane et al., 2005). During cellularization in early embryonic stage PATJ is associated with the leading edge of the invaginating membrane, although a role for this localization has not yet been established.

Due to a lack of a clean PATJ mutant, further investigation of the role of this gene in cell polarity and development of *Drosophila* was not possible. Discrepancies in the functions described for PATJ before could have risen from the use of artificial construct, like the N-terminal of PATJ to rescue deletion mutants (Nam and Choi, 2006; Pielage et al., 2003). In other reports RNAi mediated down regulation of PATJ might have resulted in off targets and dose dependent effects (Nam and Choi, 2006). Although PATJ have been studied to certain extent in *Drosophila* photoreceptor cell epidermis in the eye, the role of Crb seems to differ than in other polarized tissues like embryonic epidermis or follicle cell epithelium (reviewed

by Bulgakova and Knust, 2009). However it is likely that PATJ may function differently in different tissue types, a matter that needs to be elucidated.

## 2.5 Actin-myosin cytoskeleton

The cell cytoskeleton consists of a scaffold embedded in the external environment of cell cytoplasm. Actin polymers mainly comprises of the cytoskeleton along with other molecular motors like myosin and accessory proteins which initiate actin polymerization, control growth of actin filaments and protein turnover. Actin and myosin first discovered in the muscles makes up for half of the total protein content of the cytoskeleton. Under physiological conditions actin monomers polymerize in a spontaneous manner into polar long stable filaments where one end of the filament grows faster than the other (Pollard 2007). Through a cascade of hydrolysis of Adenosine triphosphate (ATP) to Adenosine diphosphate (ADP) the mobility of the actin filaments have been maintained. Polymerization of actin filaments give rise to certain physiological cell activities like establishing and maintaining cell morphology, cell motility, cell division and intracellular transport (Pollard and Cooper, 2009).

Interaction of actin filaments with myosin motors results in production of a force which helps the actin filaments to contract forming a cleavage producing subsequent cell division and formation of tissue architecture. On the other hand myosin motors also helps to move cargos like macromolecular complexes of RNA and proteins along actin filaments. Different types of myosin motors have been reported so far. Among them the most described ones are Myosin I, Myosin II and Myosin V. Myosin I binds to the Arp2/3 complex which helps in nucleation of actin filaments and subsequently helps in the process of endocytosis, while Myosin II (also known as non-muscle myosin) polymerizes into bipolar filaments, which can produce a contraction by pulling actin filaments together influenced by RhoGTPase (Miller at al., 2009).

## 2.6 Myosin II

Myosin II is one of the several identified motor proteins which binds to Actin filaments and control mechanistic regulation of cell migration and movement. Myosin structurally consists of six different parts: two heavy chains known as Myosin Heavy Chain (MHC) each of which contains a head domain and long coiled-coiled domains; two regulatory light chains (MRLC); two light chains known as Myosin Light Chain (MLC) which separates the head and the coiled-coiled domains (Mooseker MS et al., 1995, Foth BJ 2007). Stabilization of this hexameric structure comes through dimerization of the coiled-coiled domains. The globular head domain of MHC has an ATPase activity, whereupon ATP hydrolysis catalysed by the enzyme induces conformational change and contractility (Spudich JA 2001). Generation of contractile forces for Actin filament crosslinking requires activation of Myosin II which is achieved via phosphorylation of two conserved amino acid residues at Threonine 18 and Serine 19 of the regulatory light chain. Although Myosin Regulatory Light Chain Kinase (MRLCK) has been the principle kinase phosphorylating MRLC, other kinases like ROCK-I, ROCK-II, MRCK, PAK kinases, and citron kinase, also phosphorylate it (Aguilar-Cuenca R et al., 2014). On the other hand a trimeric complex consisting of Myosin phosphatase, a class 1 protein phosphatase (PP1cδ), a protein of unknown function and the Myosin-Binding Subunit (MBS), dephosphorylates and thereby inactivates Myosin (Matsumura and Hartshorne, 2008). Vice versa Myosin phosphatase is inactivated via phosphorylation of MBS by ROCK-I (Kawano et al., 1999).

In *Drosophila* the hexameric Myosin II complex is highly conserved. The MHC protein is encoded by the *zipper* (*zip*) gene while the MRLC is encoded by *spaghetti squash* (*sqh*). Previous studies have reported that *zip* deficient embryos have morphogenetic defects like impaired dorsal closure, head involution, and axon patterning (Young et al., 1993), while

expression of a tagged version of the *zip* protein in amneoserosa cells can restore the cortical localization (Franke et al., 2005). The process could possible mediated by cell-cell adhesion to reorganize actin along with morphogenetic forces.

*Drosophila* development has been associated with the activity of actin-myosin dynamics. Actin plays an important role in co-coordinating several events in reorganization of the cytoskeleton as the embryo starts its developmental cycle. Actin filaments condense above the nuclei at early stages but with start of the process of cellularization they are remodeled more towards the invaginating furrow where myosin II interacts with Actin filaments to form a contractile apparatus, inducing the polarized blastoderm epithelium (Warn et al., 1980 and Miller KG, Kiehart DP, 1995).

The role of Myosin II has been further implicated in the Germ Band Extension (GBE), one of the morphogenetic movements in the embryonic development. Zip and Sqh are reported to co-localize with the β-catenin (Armadilo in *Drosophila*)/E-cadherin complex during GBE in intercalating cells where the contractile acto-myosin force might regulate remodeling E-Cadherin based cell-cell contacts (Lecuit et al., 2002; Bertet et al,. 2004; Zallen and Wieschaus, 2004). On another occasion Myosin II, reported to be localized at the leading edge of the lateral epidermis during the onset of dorsal closure in late embryogenesis (Young et al., 1993).

Along with cell polarity regulators Myosin-II have been also known to regulate AJ. Rho-dependent activation of Myosin via Rok is crucial for the accumulation of E-Cadherin at cell-cell contacts thereby stabilize the AJ (Ivanov et al., 2007; Shewan et al., 2005; Yamada and Nelson, 2007). In mammalian epithelial cells as well as in the *Drosophila* epidermis, Myosin-II accumulates at the AJ (Ivanov et al., 2007; Krendel and Bonder, 1999; Shewan et al., 2005; Yamada and Nelson, 2007; Sen et al., 2012), however, activated phosphomyosin-II (measured by its phosphorylation) might not occur at all AJ but mainly at newly established ones (Yamada and Nelson, 2007).

## 2.7 The PAR complex

One of the important regulators of apical-basal polarity is the PAR-aPKC (partitioning defective– atypical protein kinase) complex. Along with the Crb complex it forms the apical domain in establishing polarity in different polarized cell types. It is highly conserved throughout evolution from worm to man (Suzuki and Ohno, 2006). The PAR complex consists of three core proteins namely the scaffolding protein PAR-3 (Bazooka (Baz) in *Drosophila*), PAR-6 and the serine-threonine kinase aPKC. Except the core components of the PAR complex, a small GTPase Cdc42 also has been reported to indirectly bind to the PAR complex. On the onset of epithelial polarization, PAR-3 associates itself with the PAR-6/aPKC hetero-dimer via PDZ domain interactions (Lin et al., 2000; Suzuki et al., 2001; Joberty et al., 2000). PAR-6 interacts with aPKC via the PB1 domains of both the proteins while the semi-CRIB domain of PAR-6 associates with Cdc42. Upon binding of Cdc42, aPKC is activated and phosphorylates PAR-3 which leads to the release of the PAR-6/aPKC complex from PAR-3 (Horikoshi et al., 2009). In Drosophila, the PDZ domain of PAR-6 was shown to bind to Crb resulting in the release of Baz from the trimeric complex (Lemmers et al., 2004).

## 2.8 PAR-6

PAR-6, a core member of the PAR/aPKC complex is known to bind to aPKC and to PAR-3 as PAR-6/aPKC heterodimer. PAR-6 consists of three distinct domains: the PDZ binding domain by which it interacts with PAR-3/Baz, the PB1 binding domain interacting with aPKC and semi CRIB *Drosophila* domain which associates with Cdc42. The binding of PAR-6 to aPKC modulates its kinase activity and thereby regulates cell polarity in various tissues

(Suzuki and Ohno, 2006). PAR-6 has been found to interact with both the known apical complexes: the PAR/aPKC and the Crb complex. The PDZ domain of PAR-6 binds to the N-terminal region of Sdt/Pals1 or the C-terminus of Crb/CRB3 ( Hurd et al., 2003; Lemmers et al., 2004; Wang et al., 2004). On the other hand the N-terminus of PAR-6 interacts with the third PDZ domain of *Drosophila* PATJ (Nam and Choi, 2003). Thus PAR-6 mediates interplay between the two known protein complexes localized to the apical domain of a polarized cell. In *Drosophila* epithelial cells Crb is required for the apical localization of PAR-6 (Kempkens et al., 2006), while in case of mammalian epithelial cells the dominant homologue of Crb, CRB3 is able to recruit PAR-6 in unpolarized cells (Hurd et al., 2003). New study reported of a WD40 protein Morg1 (mitogen-activated protein kinase organizer 1) to be a potential interaction partner of PAR-6 and also CRB3 simultaneously thereby regulating the translocation of PAR-6/aPKC to the apical junctions in MDCK (Madin-Darby-canine-kidney) cells (Hayase et al., 2013). In a separate study PAR-6 phosphorylation by aPKC induces epithelial to mesenchymal transition (EMT), a canonical pathway to tumorigenesis. (Gunaratne A, Guglielmo GM, 2013).

## 2.9 Research Objectives

Over the last decade research in cell polarity have spanned in various domains of cell function. Among them apical-basal polarity is worth mentioning as different apical and basal cues have been explored regulating the establishment and maintenance of the polarity in polarized epithelial cells. In the apical domain Crb complex forms an important cluster. Although many studies have been reported on two members of the Crb complex, Crb and Sdt, little was known with contradictory results about the third member of the complex, PATJ. Hence it is necessary to clarify the roles of PATJ in context of cell polarity and probable other functions.

In order to achieve this aim, a null mutant of PATJ has been created in *Drosophila* where the whole open reading frame of the gene has been deleted. Immuno-localization studies on embryonic epithelium and follicle cell epithelium in ovaries have shown that PATJ is not crucial for the establishment or stability of apical-basal polarity. Instead it plays a role in modulating Myosin-II dynamics by regulating Myosin-II phosphorylation and by direct binding to its regulatory light chain.

Secondly we analyzed the function of different conserved domains of PATJ. Here we found that the L27 domain is most important to the protein's function and the PDZ domains act in redundancy along with the L27 domain. Further studies also reveals that PATJ, as known before a part of the Crb complex can also form a complex with Baz and Sdt by which it gets translocated to the apical junctions. Additionally through expression of chimeric proteins we show that binding of PATJ to both Baz-Sdt and Crb-Sdt complex is necessary in embryonic epithelium.

Finally on deciphering the mechanism of how PATJ shuffles between the two complexes, PAR-6 has been found to play a central role. In oppose to the known facts that PAR-6 binds directly to Crb and Sdt ( Hurd et al., 2003; Lemmers et al., 2004; Wang et al., 2004), we

found that PAR-6 stabilizes the Crb-Sdt complex through the selective inhibition of degradation of Sdt via proteosomal linkage. PAR-6 has been found before to interact with the proteosomal receptor Rpn13 and we found that downregulation of Rpn13 or core components of the proteasomal pathway in *PAR-6*-mutant cells rescues Sdt degradation and localization..

# 3. Results

Every chapter of the results with a short description of:

- the main aim of the particular manuscript in context of the complete thesis
- the authors and their contribution to the work
- the status of the manuscript

## 3.1 PATJ localization and function in *Drosophila* is regulated by two distinct apical complexes

This project aims mainly at the structural-functional analysis of the multiple PDZ containing protein PATJ. With the use of ubiquitin promoter to express proteins close to the endogenous levels, various deletion constructs of the protein have been studied in the context of the localization pattern and functionality of the truncated proteins in rescuing the *PATJ*-null mutant allele. Further to elucidate the upstream regulators which are responsible for proper localization of PATJ, rescue experiments are performed with chimeric PATJ, able to bind Baz and Crb at the same time. The interaction of PATJ with Baz and Crb has been shown through biochemical assays.

**Arnab Sen** and Michael P. Krahn

Author contribution to work:

**Arnab Sen:** All experiments and partly writing of the manuscript

Michael P. Krahn: Editing of the manuscript

**Status: In revision** at Molecular Biology of Cell (MBoC)

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# PATJ localization and function in *Drosophila* is regulated by two distinct apical complexes

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Running title: Upstream regulators of PATJ

**Abbreviations List**

AJ, Adherens Junctions; aPKC, atypical protein kinase C; Baz, Bazooka; CR, conserved region; Crb, Crumbs; DE-Cad, *Drosophila* E-cadherin; Dlg, Discs Large; PATJ, Pals1-associated tight junction protein; Sdt, Stardust; TJ, Tight Junctions; Yrt, Yurt.

**Abstract**

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 Sdt is essential for the correct subcellular localization of PATJ in maturing epithelial cells but not during cellularization. Consistently the L27-domain of PATJ is crucial for the correct localization and function of the protein. We further demonstrate that the four PDZ domains of PATJ function to a far extent in redundancy regulating the protein's function.

Interestingly the PATJ-Sdt heterodimer is not recruited to the apical cell-cell contacts by binding to Crb but depends on functional Bazooka (Baz). Using chimeric proteins we demonstrate that the association of PATJ with both complexes, the Baz-Sdt and the Crb-Sdt complex, is crucial for PATJ's function during development of *Drosophila*.

**Highlight summary**

The conserved multiple PDZ-domain containing protein PATJ is recruited to the apical cell-cell contacts by the cell polarity regulators Crumbs and Bazooka. Indirect binding to both proteins via the adaptor molecule Stardust is necessary to accomplish PATJ's function during development of *Drosophila*.

## Introduction

Apical-basal polarization of epithelia is regulated by conserved complexes determining the apical versus the basolateral domain (Tepass, 2012; Roignot et al., 2013): At the apical tip of the lateral plasmamembrane, the PAR(partitioning-defective)-aPKC(atypical protein kinase C)-complex regulates assembly of the Crumbs(Crb)-complex, whereas the activity of these two complexes is counterbalanced by Scribble-Lethal(2) Giant Larvae-Discs Large(Dlg) which localize to the basolateral domain. Recently, 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 et al., 2003; Nam and Choi, 2003; Penkert et al., 2004; Sotillos et al., 2004; Wang et al., 2004; Kempkens et al., 2006; Krahn et al., 2010a).

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 et al., 2000; Nam and Choi, 2006; Richard et al., 2006). Recently, we and others reported that loss of PATJ in *Drosophila* epithelia does not affect apical-basal polarity in the embryonic epidermis or in follicle epithelial cells but rather modulates Myosin activity to support Adherens Junction (AJ) stability (Penalva and Mirouse, 2012; Sen et al., 2012; Zhou and Hong, 2012). Only 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 by directly stabilizing this complex or indirectly by regulating photoreceptor morphology/development (Sen 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: Beside an N-terminal L27 domain they exhibit several PDZ domains (DmPATJ four, mPATJ ten, MUPP1 thirteen) and localize to the Tight Junctions (TJ) in mammalian epithelial cells (Adachi et al., 2009). However, Abachi et al. showed that

despite its domain similarity, mPATJ but not MUPP1 regulates TJ stability (Adachi 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 et al., 2005; Shin et al., 2005). Other studies describe a role of mPATJ in Myosin-driven processes like apical constriction and cell migration (Shin et al., 2007; Ernkvist et al., 2009; Nakajima and Tanoue, 2011).

In this study we report that in the embryonic epidermis of *Drosophila* PATJ can be found in the described Crb-Sdt complex but additionally associates with the Baz-Sdt-complex we described previously (Krahn et al., 2010a). Notably deletion of Baz and Sdt but not of Crb leads to mislocalization of junctional PATJ during gastrulation and in mature epithelia of the embryonic epidermis. In contrast, localization of PATJ at the tip of the invaginating plasmamembrane during cellularization is independent of Baz/Sdt. Consequently, deletion of the L27-domain of PATJ leads to an abolished junctional accumulation and impaired function of the protein. Studies with chimeric proteins further suggest that binding to the Baz-(Sdt) complex as well as to the Crb-(Sdt) complex are inevitable for PATJ's function. Finally we investigated the functionality of PATJ's four PDZ domains and demonstrate that under close to endogenous expression levels, these domains function partly in redundancy.

## 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 et al., 2012). Studies in *Drosophila* and cultured mammalian epithelial cells proposed that PATJ associates with Sdt/Pals1 which in turn binds to the transmembrane protein Crb which targets the complex at the TJ in vertebrates and in the corresponding "subapical region" in *Drosophila* (Klebes and Knust, 2000; Roh et al., 2002).

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 mature epithelial cells when Crb is absent (Krahn et al., 2010a). 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 available to stabilize the Crb complex (Krahn et al., 2010a). We therefore tested whether the subcellular localization of PATJ is dependent on Crb or Baz or both. In *crb*-mutant embryos, PATJ shows a normal localization not only during cellularization (data not shown) but also after gastrulation as long as apical-basal polarity is still intact (stage 6-9, Fig. 1A, B). Only in later stages (from stage 10/11 on), apical-basal polarity is impaired upon loss of Crb, finally resulting in a multilayered epithelium. Here, PATJ is cytoplasmic or in aggregates (Fig. 1C). Notably, loss of cortical PATJ in these embryos is accompanied by a loss of membrane-associated Baz (Fig. 1C).

In contrast, in maternal and zygotic *baz* mutant embryos ($baz^{815-8}$ germ line clones), accumulation of PATJ at the tip of the furrow canal during plasma membrane invagination is not affected (Fig 1D) but targeting of the protein to the apical junctional region after cellularization is abolished (Fig. 1E).

Furthermore, we found endogenous PATJ and Sdt to coimmunoprecipitate with endogenous Baz in lysates from wild type embryos (Fig. 1G). Consequently, in embryos lacking Sdt, PATJ is correctly localized during cellularization (data not shown) but fails to relocalize to the apical AJ during gastrulation (Fig. 1F), indicating that PATJ is recruited by Sdt to the apical junctions. This is consistent with studies in cultured mammalian cells demonstrating that PATJ directly binds to Pals1 via hetero-dimerization or even hetero-oligomerization of its L27 domain with the (more N-terminal) L27 domain of Pals1 (Roh et al., 2002; Li et al., 2004; Feng et al., 2005). Beside its association with Baz-Sdt, PATJ can also be co-immunoprecipitated with Crb-GFP expressed from its endogenous promoter (Klose et al., 2013, Fig. 1H), pointing to a second complex, consisting of Crb-Sdt-PATJ, which might be formed later in development as soon as Sdt is released from Baz upon phosphorylation by aPKC (Krahn et al., 2010a). However the fact that PATJ remains correctly localized in the absence of Crb even in later stages (stage 8/9) indicates that Baz can complement Crb's function regarding junctional targeting of Sdt/PATJ.

**PATJ localization in the follicular epithelium depends on Sdt, Baz, and partly on Crb**

Similar to the embryonic epidermis loss of Sdt in the epithelial cells surrounding the oocyte (follicular epithelium) abolishes apical accumulation of PATJ (Fig 2A, mutant clones are marked by the absence of RFP). In *baz*-mutant clones, Sdt as well as PATJ are lost from the apical junctions (data not shown and Fig. 2B, mutant clones are marked by the absence of RFP and Baz staining, note that the follicular epithelium becomes partly multilayered (arrow)). Notably, in *crb*-defective follicle cells, apical Sdt and PATJ staining is drastically diminished but a minor fraction of the protein still accumulates apically (Fig. 2C, arrow), although it is unclear whether this is the primary consequence of loss of Crb or the result of impaired Baz localization, which is affected upon removal of Crb in the follicular epithelium, too (Fig. 2D). Thus, the follicular epithelium represents an intermediate phenotype between the epidermis (PATJ localization is only dependent on Baz-Sdt but not on Crb-Sdt) and pupal

photoreceptor cells, where PATJ localization depends on Crb (Richard et al., 2006). Vice versa in photoreceptor cells, PATJ seems to be crucial for the stabilization of the Crb-Sdt complex (Nam and Choi, 2006; Richard et al., 2006; Zhou and Hong, 2012), whereas this phenotype is much weaker in the follicular epithelium and not seen at all in the embryonic epidermis: 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 (Penalva and Mirouse, 2012; Sen et al., 2012).

**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. 3A). To avoid artificially increased protein levels, we expressed the modified proteins under a ubiquitous promoter (Ubiquitin) and used the PhiC31-Integrase system (Groth et al., 2004) to generate transgenic lines with identical genomic background, ensuring comparable protein levels. Indeed, wild-type PATJ-GFP expressed in this system is expressed at similar levels as endogenous PATJ (Fig. 3B), localizes indistinguishable from endogenous PATJ (Fig. 3C) and is capable to rescue the $PATJ^{\Delta 1}$ null allele (79% surviving flies, Fig. 3A).

In mammalian epithelial cells, mPATJ has been shown to be targeted by Pals1 to the TJ via a heterodimerization of their L27 domains (Roh et al., 2002; Li et al., 2004; Straight et al., 2004). Likewise, deletion of the L27 domain of *Drosophila* PATJ results in a cytoplasmic accumulation of the mutant protein in the embryonic epidermis as well as in follicle cells (Fig. 3D and data not shown). Consequently, the $PATJ_{\Delta L27}$-GFP is unable to rescue a *PATJ*-null allele, resulting in similar phenotypes as the null allele ($PATJ^{\Delta 1}$, pupal lethality).

In contrast to deletion of the L27-domain, removal of any of the four PDZ domains alone does not impair the subcellular localization of the modified protein at the apical junctions (data not shown). Furthermore, ubiquitous expression of all single deletion constructs can complement

for PATJ's function and can be maintained as a stable stock with the homozygous *PATJ*$^{\Delta 1}$ allele. However, analysis of the hatching rates showed that deletion of the first PDZ-domain affects functionality of the protein far more than deletion of PDZ2, 3 or 4 (34% in comparison to 58, 55, 68%, respectively, Fig. 3A).

As a truncated version of PATJ has been reported to be capable to partly rescue a PATJ-mutant (Nam and Choi, 2006; Richard 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 junctional localization, although not as delimited as the wild-type protein (Fig. 3E). This protein, lacking all PDZ domains, shows no rescue capacity. Experiments with flies lacking zygotic PATJ expression and ubiquitously expressed $PATJ_{1\text{-}240}$-GFP (L27 domain and the first PDZ domain) produced occasionally adult flies. However the majority of flies died during late pupal stages but in contrast to the null allele, pupae in the $PATJ_{1\text{-}240}$ rescue undergo complete morphogenesis and die only shortly before hatching (or fail to hatch). Hatched flies are 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 is not capable to fully replace the wild-type protein. Overexpression of the same construct using arm::GAL4 resulted in increased rescue capacity and the recued 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 function of PATJ during development, which is in line with previous studies using overexpressed proteins (Nam and Choi, 2006; Penalva and Mirouse, 2012).

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

These results suggest that none of the PDZ domains is inevitable for the proteins function but that they function in redundancy and under overexpression conditions, the first PDZ-domain is sufficient for viability of the fly. This is further supported by the observation that upon deletion of the first two PDZ domains ($PATJ_{\Delta PDZ1+2}$) or the first and the fourth PDZ domain ($PATJ_{\Delta PDZ1+4}$) the mutated protein can still rescue $PATJ^{\Delta 1}$. However, survivor rates (Fig. 3A) indicate that deletion of more than one PDZ domain strongly reduces PATJ's functionality. Thus the multiple PDZ domains of PATJ might contribute to its physiological function and further enhance junctional recruitment of PATJ as under endogenous expression levels the isolated L27 domain shows a certain cytoplasmic mislocalization which is not observed in constructs compromised of several PDZ domains (data not shown).

Taken together, our data revealed a surprising redundancy of the PDZ domains during *Drosophila* development. This fact is even more unusual as all four PDZ-domains share only 50-60% identity and similar amino acids between each other.

**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. 4A). Notably the localization of this chimeric protein is more or less cytosolic with only a minor fraction accumulating at the apical junctions (Fig. 4B). This might be due to the fact that Sdt-levels are restrictively controlled: Even moderately increased protein levels lead to an entirely cytosolic localization (data not shown). Nonetheless $PATJ_{\Delta L27\text{-}PDZ(Sdt)}$ restores to some extent the rescue-capacity of the protein (19% hatching flies, Fig. 4A). The addition of the PDZ domain of PAR6, which is capable to directly bind to both, Baz (Joberty et al., 2000; Lin et al., 2000) and Crb (Lemmers et al., 2004; Kempkens et al., 2006) to $PATJ_{\Delta L27}$ results in a more junctional localization of the chimeric protein, although a substantial amount is still cytosolic (Fig. 4C). $PATJ_{\Delta L27}$-

$_{PDZ(PAR6)}$ rescues the PATJ null allele similar to $PATJ_{\Delta L27\text{-}PDZ(Sdt)}$ (13% hatching flies, Fig. 4A). In contrast, a protein composed of the four PDZ-domains of PATJ and a fragment of Baz which accumulates at the apical junctions by direct binding to the plasmamembrane (Krahn et al., 2010b) is to a far extent correctly targeted to the apical junctions ($PATJ_{\Delta L27\text{-}LB(Baz)}$, Fig. 4D) but does not rescue the *PATJ*-null allele (Fig. 4A).

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

As outlined above, Baz is essential to initially recruit Sdt to apical junctions –in later stages, this complex is (in part) released by phosphorylation of Baz by aPKC, resulting in apically enriched Sdt which is capable to stabilize Crb. To dissect, whether PATJ exhibits its function through a Baz-Sdt or via a 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 et al., 2006) or a Baz-binding domain (oligomerization domain CR1, Benton and Johnston, 2003; Desai et al., 2013).

Interestingly although $PATJ_{\Delta L27\text{-}CR1(Baz)}$ and $PATJ_{\Delta L27\text{-}FERM(Yurt)}$ localize to a great extent correctly at the apical junctions (Fig. 4E-F) none of these chimeric proteins is capable to rescue $PATJ^{\Delta 1}$ (Fig. 4A). Thus, association of PATJ with both complexes, Baz-Sdt and Crb-Sdt is essential for the proteins function. This might be explainable by the implication of PATJ in regulation of the cytoskeleton: By modulating Myosin-Phosphatase PATJ regulates Myosin activity which is essential for several morphological processes, including metamorphosis. Baz in turn associates with the AJ (Harris and Peifer, 2005; Bulgakova et al., 2013) which anchors Actin-Myosin filaments as well as Myosin-modulating enzymes (Shewan et al., 2005; Yamada and Nelson, 2007). On the other hand, Crb has been described to link the Actin-Cytoskeleton via Moesin and $\beta_{heavy\text{-}chain}$ spectrin to the plasmamembrane (Medina et al., 2002). Therefore PATJ seem to be indispensible in both complexes to

modulate Myosin dynamics in different compartments of the apical junctional region during metamorphosis.

## Materials and Methods

### *Drosophila* genetics

The following mutant alleles were used: $PATJ^{\Delta 1}$ (Sen et al., 2012), $baz^{815-8}$ (McKim et al., 1996; Krahn et al., 2010b), $sdt^{K85}$ (Berger et al., 2007) and $crb^{11A22}$ (Jürgens et al., 1984). Germ line clones were generated with the mutant alleles recombined with FRT using dominant female sterile technique (Chou et al., 1993). Homozygous mutant embryos were identified using GFP- and RFP-marked Balancer chromosomes. 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 et al., 2012) as template. The following oligonucleotides were used for mutagenesis:

PATJΔL27: 5'-GCGGATATTTCCAGCTCCATGTTGCCCAAC-3'

PATJΔPDZ1: 5'-GCCATAGAGCTGGTCCGTCCCGTTGAGCAG-3'

PATJΔPDZ2: 5'-GAAACGGAGAAGCTTCGCTACCTGAGGGGC-3'

PATJΔPDZ3: 5'-GGCTCCGATGTGGAGTGCGGTCGCAACAGG-3'

PATJΔPDZ4: 5'-ATGTGGTCGTCCCAACGCATTGGTGTGGCC-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.

Similar the PDZ domain of PAR6, the FERM domains of Moe and Yrt and the CR1 domain of Baz were cloned into NotI of PATJ 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'-GCGGCCGCTTTGACCGGCGCCCTAA-3'; Baz-CR1-F: 5'-GCGGCCGCCCCCTTCACCATGAAGGTCACCGTCTGCTTCGGC-3', Baz-CR1-R: 5'-GCGGCCGCATCTCCGCCTCCTTGC-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 et al., 1999), 2 µl of guinea pig anti PATJ (Sen et al., 2012) or 2 µl of the corresponding preimmune sera were added to 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 in 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, DSHB), guinea pig anti PATJ (1:1000, Sen et al., 2012), mouse anti Sdt (1:20, Berger et al., 2007), rabbit anti Baz (1:2000, Wodarz et al., 1999).

**Immunohistochemistry**

Embryos were fixed in 4% formaldehyde, phosphate buffer pH 7.4 as described before (Krahn et al., 2009). Primary antibodies used for indirect immunofluorescence were as follows: Guinea pig anti PATJ (1:500, Sen et al., 2012), mouse anti Sdt (1:20, Berger et al., 2007), rabbit anti Baz (1:1000, Wodarz et al., 1999), mouse anti Crb (Cq4, 1:50, DSHB), mouse anti Dlg (1:50, DSHB), rat anti DE-Cad (1:50, DSHB), rabbit anti GFP (#A11122, 1:1000, Life technologies). 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.

**Author contributions**

Arnab Sen and Michael P. Krahn performed the experiments and wrote the manuscript.

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**Figure legends**

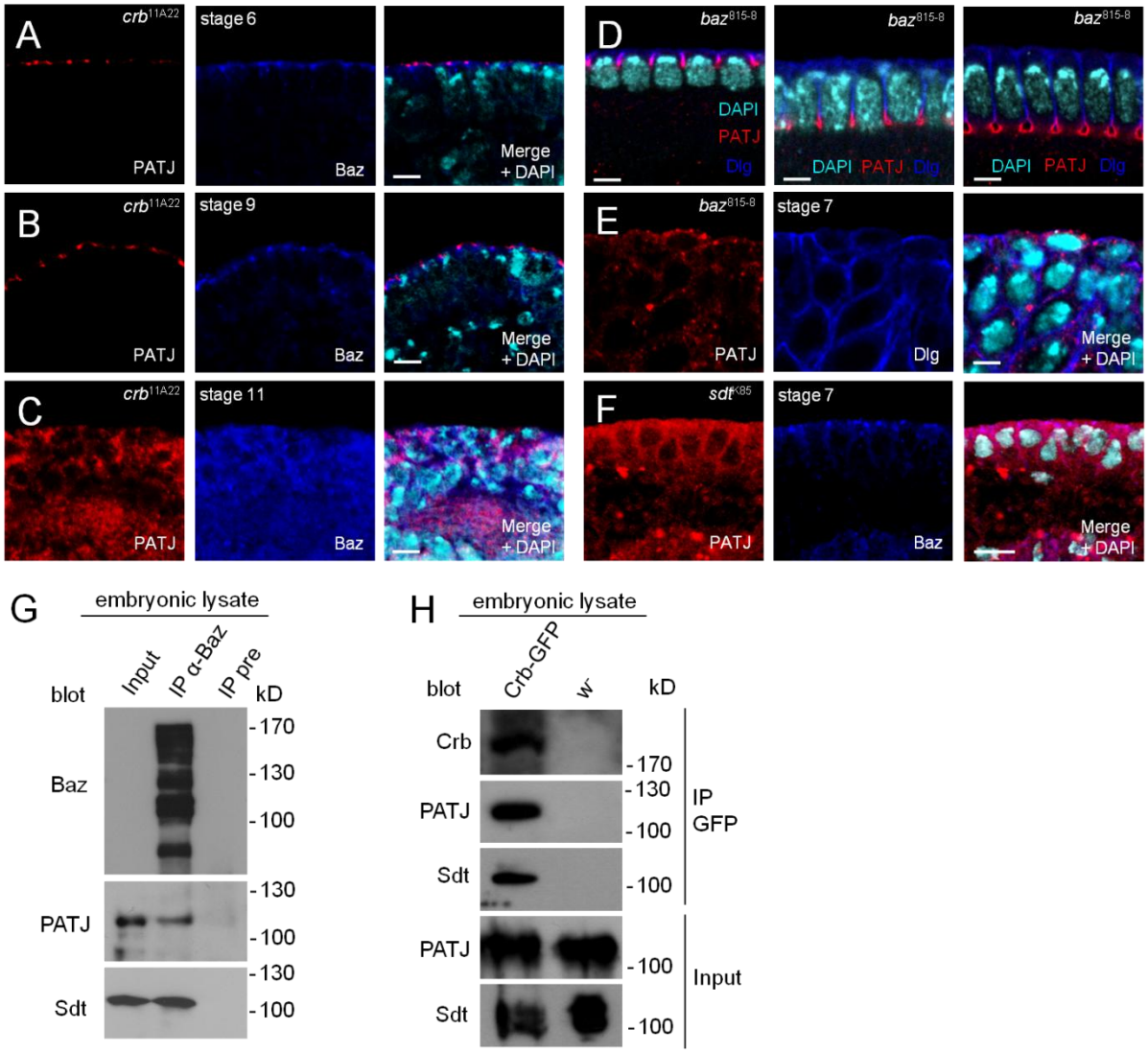
**Fig. 1. Apical junctional localization of PATJ depends on Baz and Sdt.** (A and B) during early embryogenesis. (C) Upon disruption of epithelial integrity in *crb*-mutant embryos of later stages, Baz as well as PATJ are mislocalized to the cytoplasm. (D) PATJ localization during cellularization is not affected in *baz*-mutant embryos. (E) Loss of *baz* in gastrulation results in a disturbed apical-basal polarity and cytoplasmic PATJ localization. (F) PATJ is not recruited to the apical junctions in the absence of Sdt. (G and H) Endogenous PATJ coimmunoprecipitates with Baz and Crb-GFP from embryonic lysates. Scale bars = 5µm.

**Fig. 2. Apical junctional localization in the follicular epithelium**. (A) PATJ is lost from the apical junctions in *sdt*- (A) and *baz*- (B) mutant clones but is partly retained in *crb*-mutant clones (C). Mutant cells are marked by the absence of RFP (A and B) or GFP (C), respectively. (D) In *crb*-mutant follicle cells, localization of Baz to the apical junctions is diminished. Scale bars = 10µm.

**Fig. 3. Structural-functional analysis of PATJ**. (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 flies indicates that PATJ-GFP is expressed at similar levels as the endogenous protein. PATJ-GFP (C) localizes to the apical junctions indistinguishable from endogenous protein, whereas as 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. Scale bars = 5µm.

**Fig. 4. Dissection of essential upstream complexes of PATJ**. (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-F) Subcellular localization of chimeric proteins described in A. Scale bars = 5µm.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PATJ
stage 6
Baz
Merge + DAPI
B
crb11A22
PATJ
stage 9
Baz
Merge + DAPI
C
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PATJ
stage 11
Baz
Merge + DAPI
D
baz815-8
DAPI
PATJ
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E
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Input
IP α-Baz
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170
130
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**Figure 1**

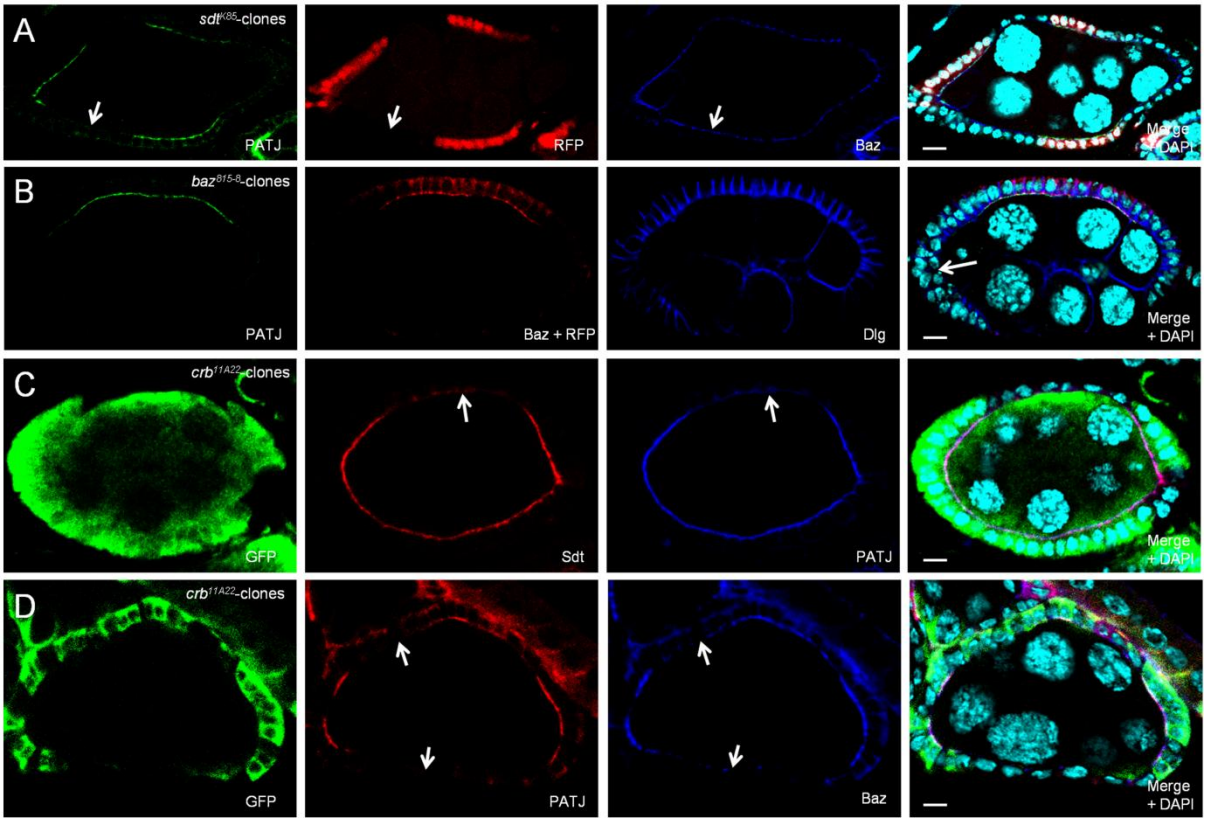


**Figure 2**

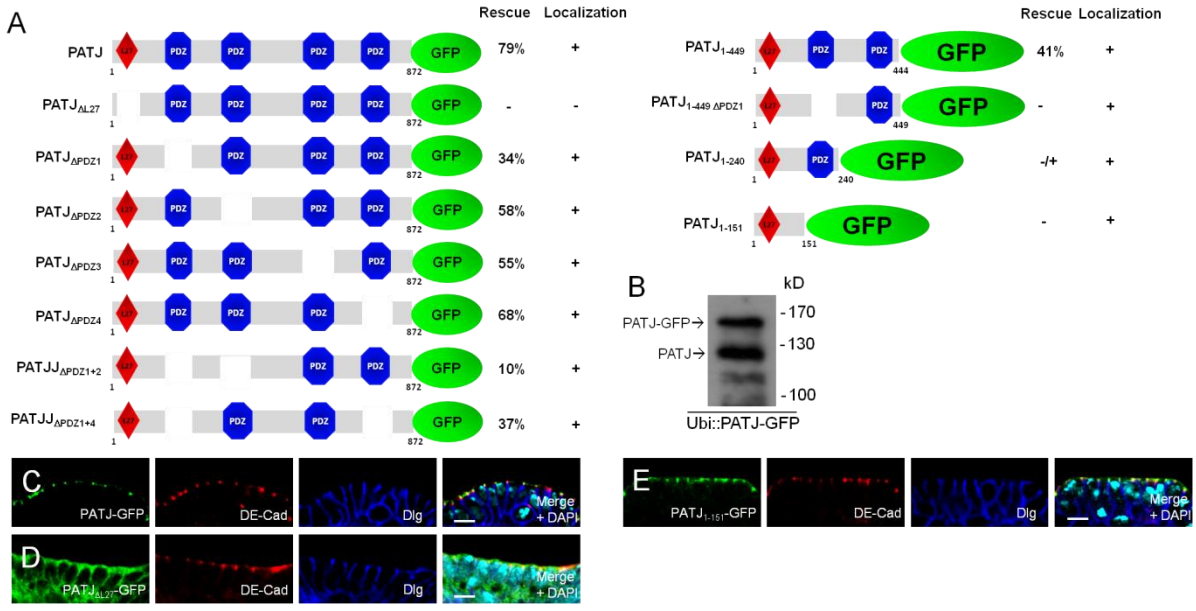


**Figure 3**

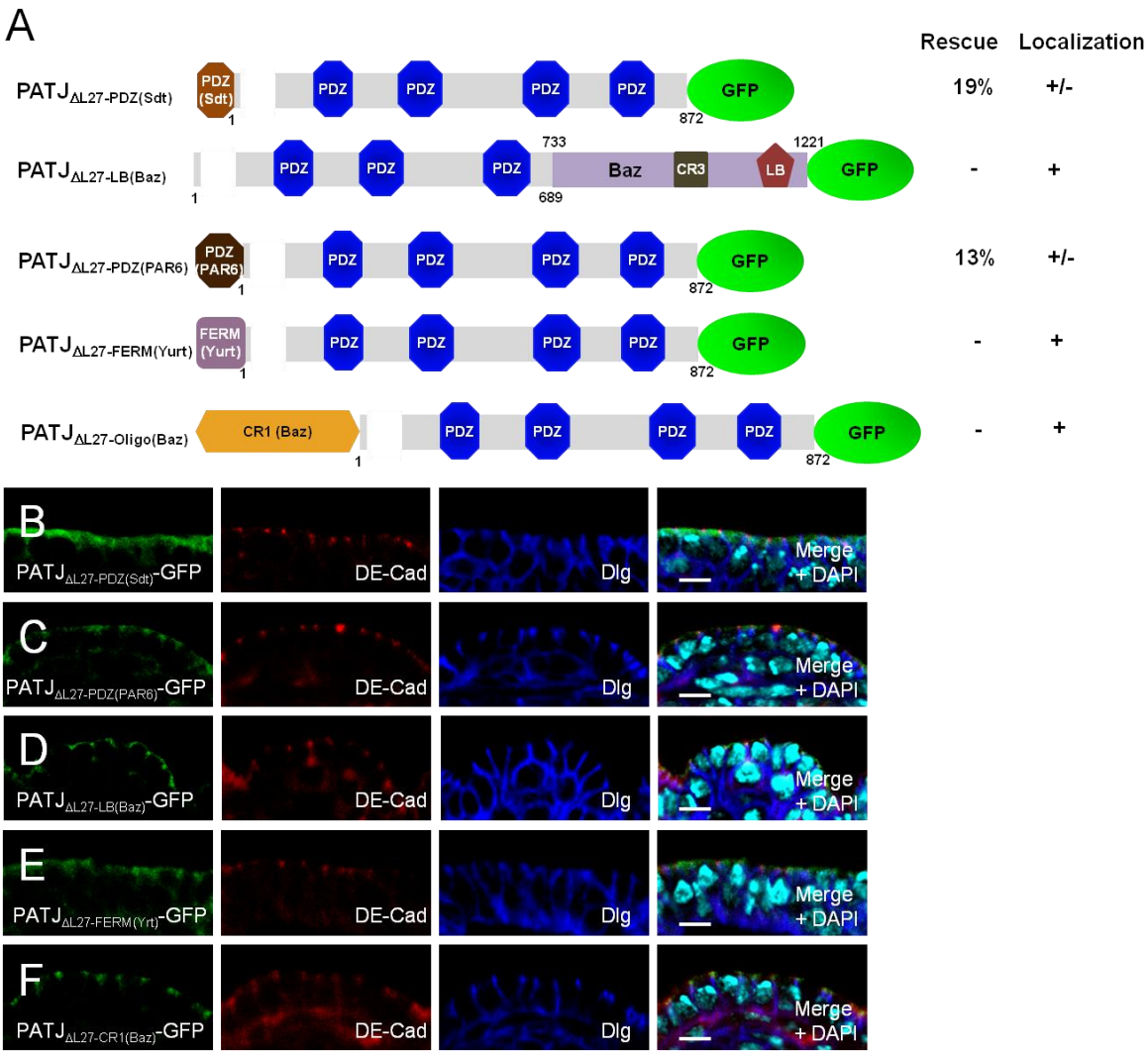
A
Rescue
Localization
PATJ ΔL27-PDZ(Sdt)
PDZ (Sdt)
PDZ
GFP
1
872
19%
+/-
PATJ ΔL27-LB(Baz)
733
1221
Baz
CR3
LB
689
-
+
PATJ ΔL27-PDZ(PAR6)
PDZ (PAR6)
13%
PATJ ΔL27-FERM(Yurt)
FERM (Yurt)
PATJ ΔL27-Oligo(Baz)
CR1 (Baz)
B
PATJ ΔL27-PDZ(Sdt)-GFP
DE-Cad
Dlg
Merge + DAPI
C
PATJ ΔL27-PDZ(PAR6)-GFP
D
PATJ ΔL27-LB(Baz)-GFP
E
PATJ ΔL27-FERM(Yrt)-GFP
F
PATJ ΔL27-CR1(Baz)-GFP

**Figure 4**

## 3.2 *Drosophila* PATJ supports adherens junction stability by modulating Myosin Light Chain activity

In this work, we investigated the function of PATJ in the context of apical-basal polarity. We created a *PATJ*-null mutant to check the localization of other proteins known to localize at the apical junctions of epithelial cells through immuno-cytochemistry. Through genetic interaction experiments, co-immunoprecipitation and GST pull down assays we have proven that PATJ directly binds to and modulates the phosphorylation of Myosin-II, found in a mass spectrometry screen before.

Further we also found that PATJ stabilizes Myosin-II in presence of weak Adherens Junction, thereby functions in the morphogenesis and stability of cell-cell junctions.

**Arnab Sen**, Zsanett Nagy-Zsvér-Vadas and Michael P. Krahn

Author contributions to work:

**Arnab Sen:** All experiments except the genetic interaction studies, partly writing of the manuscript

Zsanett Nagy-Zsvér-Vadas : Genetic interaction studies

Michael P. Krahn: Editing of the manuscript

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# *Drosophila* PATJ supports adherens junction stability by modulating Myosin Light Chain activity

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**Abstract**

The assembly and consolidation of the Adherens Junctions (AJ) are key events in the establishment of an intact epithelium. However, AJ are further modified to obtain flexibility for cell migration and morphogenetic movements. Intact AJ in turn are a prerequisite for the establishment and maintenance of apical-basal polarity in epithelial cells. In this study, we report that the conserved PDZ-domain containing protein PATJ (Pals1-associated tight junction protein) was not *per se* crucial for the maintenance of apical-basal polarity in *Drosophila* epithelial cells but rather regulated Myosin localization and phosphorylation. PATJ directly bound to the Myosin Binding Subunit of Myosin Phosphatase and decreased Myosin dephosphorylation, resulting in activated Myosin. Thereby PATJ supports the stability of the *Zonula Adherens*. Notably, weakening of AJ in a *PATJ*-mutant epithelium led first to a loss of Myosin from the AJ, subsequently to a disassembly of the AJ and finally to a loss of apical-basal polarity and disruption of the tissue.

## Introduction

The establishment and maintenance of cell polarity in epithelial cells is closely connected with the formation of cell-cell junctions. Notably, most of the key players regulating both processes have been highly conserved throughout evolution, ranging from worm to men. In the Adherens Junction (AJ) belt, trans-dimerization of the extracellular domain of Cadherins from adjacent cells enforced by lateral clustering of Cadherins expressed on the same cell mechanically link neighboring cells. To accomplish a robust anchorage to the cytoskeleton, the intracellular tails of Cadherins are dynamically linked via adaptor proteins of the Catenin family to Actin filaments, resulting in an adhesive belt-like structure (Nelson, 2008). The correct assembly of AJ in turn is required for the clustering of transmembrane proteins (e.g., Claudins and Occludins) and their cytoplasmic adaptors (e.g., *Zonula Occludens* proteins) more apically, which leads to the formation of the Tight Junctions (TJ) (Chiba et al., 2008; Shin et al., 2006). Thereby the intercellular space is efficiently sealed and an intramembranous diffusion barrier is established, dividing the plasma membrane into an apical domain and a basolateral domain.

In addition to the mentioned transmembrane proteins, two protein complexes localize to the TJ: First, the transmembrane protein Crumbs (Crb) with its intracellular adaptor protein Pals1 (Protein associated with Lin seven 1, Stardust (Sdt) in *Drosophila*), which in turn recruits PATJ and Lin-7 to the cortex (Bulgakova and Knust, 2009). Second, the scaffolding protein PAR-3 (Bazooka (Baz) in *Drosophila*) targets and the atypical Protein Kinase C (aPKC) to the junction (Suzuki and Ohno, 2006). Although invertebrates such as *Drosophila* do not express Occludins and therefore do not develop TJ, the components of the Crb-complex are localized to the TJ-analogues region (often addressed as “subapical region,” SAR) (Bachmann et al., 2001; Harris and Peifer, 2005; Tepass, 1996), whereas Baz in contrast concentrates at or slightly apical to the AJ (Harris and Peifer, 2005; Krahn et al., 2010). Thereby, the Crb- and Baz-complexes define the apical compartment, which is counterbalanced by the laterally

localized proteins Lethal (2) Giant Larvae (Lgl), Discs Large (Dlg) and Scribble (Bilder et al., 2003; Tanentzapf and Tepass, 2003).

Another key regulator of the AJ is the Actin-Myosin cytoskeleton itself: The hexameric, contractile non-muscle Myosin II (henceforth Myosin) crosslinks Actin filaments and consists of a homodimer of two Myosin Heavy Chain proteins (MHC, encoded by *zipper* (*zip*) in *Drosophila*), which is stabilized by two Myosin Essential Light Chain peptides (MELC, encoded by *mlc-c* in *Drosophila*) bound to the "head"- (globular) domain of MHC. In addition, MHC is regulated by two Myosin Regulatory Light Chains (MRLC, Spaghetti squash (Sqh) in *Drosophila*), which are also associated with the head-domain (Vicente-Manzanares et al., 2009).

Myosin dynamics drive many if not all morphological processes in *Drosophila*, for instance cellularization (reviewed by Mazumdar and Mazumdar, 2002), germ band extension (the elongation of the embryo, Bertet et al., 2004; Zallen and Wieschaus, 2004) or dorsal closure (Young et al., 1993) as well as cell migration in many contexts (Parsons et al., 2010; Vicente-Manzanares et al., 2009).

To transmit contractile forces, Myosin has to be activated by phosphorylation of the regulatory light chain at two conserved residues, which is accomplished mainly by Rho-associated Kinase (Rok) and Myosin Regulatory Light Chain Kinase (MRLCK). Upon phosphorylation, Actin-induced Myosin ATPase activity is increased and assembly-competence is promoted resulting in crosslinking of Actin-filaments (Vicente-Manzanares et al., 2009). Vice versa, Myosin Phosphatase, a trimeric complex of a class 1 protein phosphatase (PP1cδ), a protein of unknown function and the Myosin-Binding Subunit (MBS), dephosphorylates and thereby inactivates Myosin (Matsumura and Hartshorne, 2008). Myosin phosphatase in turn is inactivated via phosphorylation of MBS by Rok (Kawano et al., 1999). Thus Rok activates Myosin directly by phosphorylation and indirectly by decreasing Myosin dephosphorylation.

Rho-dependent activation of Myosin via Rok is crucial for the formation and stabilization of AJ (Ivanov et al., 2007; Shewan et al., 2005; Yamada and Nelson, 2007). In mammalian epithelial cells as well as in the *Drosophila* epidermis, Myosin accumulates at the AJ (Ivanov et al., 2007; Krendel and Bonder, 1999; Shewan et al., 2005; Yamada and Nelson, 2007 and this work); however, activation of Myosin (measured by its phosphorylation) might not occur at all AJ but predominately at newly established junctions (Yamada and Nelson, 2007).

Loss of Crb and Sdt/Pals1 as well as Baz/PAR-3 and aPKC/ has been shown in various systems to strongly affect apical-basal polarity in epithelial cells, finally resulting in a breakdown of the AJ and disorganization of the tissue (Fogg et al., 2005; Harris and Tepass, 2008; Harris and Peifer, 2004; Harris and Peifer, 2007; Mizuno et al., 2003; Müller and Wieschaus, 1996; Straight et al., 2004; Suzuki et al., 2002; Tepass, 1996).

In contrast, little or contradicting information is available about the third "core" component of the Crb-complex, PATJ. The domain structure of PATJ is not as conserved as the one of Crb or Sdt – besides a common L27 domain, mammalian PATJ is composed of ten PDZ-domains, whereas *Drosophila* exhibits only four. Furthermore, a second protein (MUPP1) shows a high similarity to and partly overlapping functions with PATJ in mammals (Adachi et al., 2009). Nonetheless, in both systems, PATJ has been reported to function in the establishment of cell polarity: In cultured epithelial cells, RNAi-mediated downregulation of PATJ protein results in a loss of Pals1 from the TJ and a strongly decreased assembly of the TJ (Michel et al., 2005; Shin et al., 2005). Affected cells do not fully polarize and fail to form cysts in a three-dimensional culture (Shin et al., 2005) and TJ markers such as ZO-1 and Occludin are mislocalized to the lateral membrane (Michel et al., 2005). Similar effects were observed overexpressing a dominant-negative version of PATJ in MDCK cells (Hurd et al., 2003). Interestingly, Shin *et al*. found that in wound healing experiments, PATJ localizes PAR-3 and aPKC to the leading edge, suggesting a function of PATJ in cell migration (Shin et al., 2007).

In *Drosophila*, the role of PATJ in morphogenesis and cell polarity has been discussed controversially: An initial report describing *PATJ* as the *discs lost* gene (Bhat et al., 1999) was corrected by Pielage *et al*. (Pielage et al., 2003). Although PATJ was not the focus of that report, the authors found that loss of PATJ does not affect embryonic development, but, due to a lack of a clean *PATJ* mutant, they did not follow up these findings. On the other hand, several reports indicate that in the *Drosophila* eye, PATJ is crucial for stabilizing Crb and Sdt at the stalk membrane of photoreceptor cells and for preventing light-induced degeneration of rhabdomeres (Nam and Choi, 2006; Richard et al., 2006) and regulating frizzle-dependent planar polarity (Djiane et al., 2005). In follicular epithelial cells, PATJ was found to be implicated in the control of apical-basal polarity by stabilizing the Crb/Sdt complex (Tanentzapf et al., 2000).

To clarify the role of PATJ in *Drosophila* epithelial cell polarity, we established a *PATJ* null allele. Surprisingly, PATJ deficient flies do not show obvious polarity defects and mainly die during early puparation. However, a significant proportion of mutant embryos show morphogenetic defects which can be partly rescued by overexpression of Myosin or decreased Myosin dephosphorylation. *PATJ*-mutant phenotypes are dramatically enhanced upon removal of one copy of *shotgun* (*shg*), the gene encoding *Drosophila* E-cadherin (DE-Cad), resulting in a displacement of junctional Myosin and finally leading to a disassembly of the weakened adherens junctions and loss of apical-basal polarity in the epidermis. Finally we found that PATJ directly interacts with the Myosin Binding Subunit of Myosin phosphatase and co-regulates Myosin phosphorylation and thus Myosin dynamics.

**Results**

*Drosophila* PATJ shows two distinct localization patterns during epithelial polarization.

During cellularization (the formation of single epithelial cells from a syncytium in early embryonic development) PATJ accumulates at the tip of the invaginating membrane, the so-called furrow canal (Bhat et al., 1999 and Fig. 1 A-E), colocalizing with Sqh, whereas Baz and DE-Cad assemble more apically first in the basal and later in the apical AJ (Fig. 1 A-E and data not shown). Upon maturation of the epithelium during gastrulation, PATJ is recruited to the emerging apical AJ belt, colocalizing with Baz and DE-Cad (Fig. 1 F and data not shown) and, in differentiated epithelial cells of the embryonic epidermis, PATJ localizes similarly to its mammalian homologue, to the apical tip of the lateral membrane (Fig. 1 G). Here, as well as in cells of the follicular cell epithelium, it colocalizes with Crb and Sdt (Fig. 2 E and data not shown).

Loss of PATJ results in pupal lethality

To elucidate the function of *Drosophila* PATJ in epithelial polarity, we established a *PATJ* null allele ($PATJ^{\Delta 1}$) by using homologous recombination (Huang et al., 2008). Loss of PATJ protein expression was tested by immunostainings (Fig. 2 I and J) and by Western blotting (Fig. S1 A). Around a quarter of the embryos lacking zygotic expression of PATJ do not hatch after embryogenesis; the same proportion dies in early larval stages and the rest as pupae (Fig. 2 A). Dissection of *PATJ*-mutant pupae revealed that these flies do not initiate metamorphosis and die during early pupal stages (Fig. 5 C).

Ubiquitous expression of GFP-tagged PATJ can fully rescue the lethality and all observed phenotypes (data not shown), indicating that the $PATJ^{\Delta 1}$ allele does not contain mutations in other genes and is a clear null allele.

Because of its strong maternal contribution, we generated $PATJ^{\Delta 1}$ germ line clones producing embryos lacking the maternally provided mRNA/protein and the zygotically expressed copy. Notably, these flies show nearly the same lethality pattern as their zygotic-mutant

counterparts (Fig. 2 A), exhibiting only an increased lethality in third instar larvae at the expense of dead pupae. Embryos maternally mutant for *PATJ*, which have been fertilized by wild type males, develop until adulthood and hatch without any phenotypes, indicating that the maternally provided protein is dispensable for normal development.

Although PATJ is strongly expressed early in embryonic development, staining with antibodies against Sqh, Nullo, Dlg and Slow-as-moleass (Slam) as markers for the invaginating plasma membrane during cellularization revealed no defects in this process (data not shown).

PATJ does not affect apical-basal polarity

We further analyzed apical-basal polarity in the embryonic epidermis of $PATJ^{\Delta 1}$ mutants in different developmental stages. Surprisingly, we did not detect any defects in the localization of the AJ components DE-Cad and Armadillo (Arm, the *Drosophila* homologue of β-catenin), the apical determinants Crb, Sdt, Baz, aPKC, and  and the lateral polarity proteins Dlg, α-spectrin and Coracle (Fig. 2 B and C, compare to wild type in D and E, Fig. S2 A-B and data not shown).

Although lethality and staining with cell polarity markers do not point to a crucial role of PATJ during embryogenesis, around 18% of the dead embryos display strong cuticle defects: A general shortening of the cuticle and head defects but unimpaired segmentation (Fig. 2 F and G). 46% show an unaffected cuticle and more than 30% of the dead embryos fail to develop any cuticle, presumably because they die before cuticle is secreted. Immunostainings of *PATJ*-mutant embryos produced similar results as cuticle preparations: Approximately 15% of dead embryos fail to retract the germ band correctly (Supplemental videos 1+2 showing wild type (video 1) and *PATJ*-mutant (video 2) embryos, expressing DE-Cad-GFP as plasmamembrane marker; Fig. 2 H, compare to 2 I, head regions of the embryos are marked by an arrow, end of the germ bands are marked by an arrowhead). Notably, segmentation and cell polarity are not impaired even if the overall embryonic morphology is severely disturbed

(Fig. 2 B and C), indicating that the observed morphology defects are not due to impaired apical-basal polarity. These embryos do not show increased apoptosis in comparison to wild type embryos or embryos heterozygous for *PATJ*$^{\Delta 1}$ (Fig. S1 B and B').

In contrast, in *PATJ*-mutant clones in the follicle cell epithelium, apical accumulation of Sdt as well Crb is weaker than in PATJ-expressing cells (Fig. 2 J and Fig. S1 C). However, a significant portion of these proteins is still correctly localized, and we did not observe loss of polarity or multilayering of this tissue, even if almost the entire epithelium of an egg chamber is mutant for *PATJ* (Fig. 2 J).

PATJ stabilizes Myosin at weak adherens junctions

*Drosophila* embryos undergo several morphological changes during embryogenesis, including invagination of the cell membrane (cellularization), germ band elongation and subsequent retraction, segmentation and finally dorsal closure. These processes are all accompanied by intensive modifications of the AJ as well as of the Actin-Myosin cytoskeleton, which is assumed to be the driving force for the morphological changes.

Because a certain percentage of *PATJ*-mutant embryos show defects in germ band retraction, we tested whether PATJ regulates Actin-Myosin dynamics. Staining for Sqh as well as for Zip revealed that Myosin localization and anchorage appears undisturbed in embryos failing to retract the germ-band (Fig. S2 D and data not shown). Moreover, other morphological processes, such as cellularization, germ band extension and dorsal closure are not affected in *PATJ* mutant germ line clone embryos (data not shown).

In intact epithelial cells, Myosin accumulates at the region of the AJ, colocalizing with DE-Cad (Fig. 3 A, C, Fig. S2 E and F), but it also shows a partly overlapping localization with PATJ, which stains slightly more apical at the AJ (Fig. 3 A and Fig. S2 E and F). Because AJ appear to be the anchoring point for Myosin accumulation, we investigated the role of intact AJ in a *PATJ*-mutant background on Myosin targeting. Interestingly, introduction of one copy of a strong *shg* loss-of-function allele (*shg*$^{R69}$) leads initially to a loss of Sqh and Zip from the

weakened AJ in the embryonic epidermis if PATJ is not present (Fig. 3 B and D, compare with A and C). Notably, in these cells, AJ are still intact as estimated by staining against DE-Cad and Arm (Fig. 3 B, D and E). Later on, AJ are disrupted and epithelial morphology is severely disturbed finally resulting in a multilayered epithelium and massive apoptosis (Fig. 3 H). In this tissue, cells lose their epithelial morphology and tend to round up, DE-Cad and Baz are mostly displaced into the cytosol/vesicles with only a minor protein fraction found aggregated at the membrane (Fig. 3 F). Moreover, the lateral marker Dlg is found in the cytoplasm as well as all around the plasmamembrane (Fig. 3 G), further indicating that apical-basal polarity is lost. Control embryos with intact AJ in a *PATJ*-mutant background show a wild-type distribution of Myosin, DE-Cad, Baz and Dlg (Fig. S2 A-D). Furthermore, in control embryos that are heterozygous for *shg* and *PATJ*, Myosin accumulation appears normal and AJ stay intact (Fig. 3 B and D).

These findings are in line with the observation that the frequency of cuticle phenotypes as well as the lethality rate of *PATJ*-mutant embryos is strongly increased upon removal of one copy of *shg* (Fig. 3 I).

PATJ associates with the Myosin Binding Subunit of Myosin phosphatase

We next investigated a potential interaction between PATJ and Myosin (dynamics). The phenotypes observed in *PATJ*-mutant embryos suggest that PATJ does not play an essential role in regulating Actin-Myosin dynamics under physiological conditions. However, upon weakening of the AJ belt, PATJ is crucial for the maintenance of Myosin accumulation at the apical cell contact zone. This might be accomplished in several ways: First by stabilizing Myosin in the apical junctional compartment by targeting or activating the Myosin modulating machinery. Second, PATJ might directly recruit Myosin filaments to the AJ or apical junctional region. Third, PATJ could influence Myosin stability or dynamics by influencing Myosin phosphorylation. The latter possibility is suggested by the fact that mammalian PATJ was found in a mass-spectrometry approach to associate with the Myosin

Binding Subunit (MBS) of the Myosin phosphatase (Ewing et al., 2007). In *Drosophila*, loss of MBS results in an overactivation of Myosin and cell motility defects in the eye and during dorsal closure (Lee and Treisman, 2004; Mitonaka et al., 2007; Mizuno et al., 2002; Tan et al., 2003).

Indeed we verified that PATJ can directly bind to MBS in vitro and associates with MBS in transfected Schneider 2R+ (S2R+) cells and under endogenous conditions in embryonic lysates (Fig. 4 A-C). In contrast to the Myosin-kinase Rok, which colocalizes with PATJ and Myosin at the cellularization front and later at the AJ (Simoes Sde et al., 2010), MBS is present only in the apical region of newly formed epithelial cells during cellularization (Fig. 4 D). In mature epithelial cells, MBS localizes in the apical cytoplasma and at the free apical membrane but is slightly enriched at the apical cell junctions, overlapping with PATJ localization (Fig. 4 E, arrows). Thus, in mature epithelial cells, PATJ might locally enhance or inhibit Myosin phosphatase by targeting or sequestering its binding subunit (MBS) at the apical junctions in mature epithelial cells. However, PATJ is not (or at least not exclusively) responsible for the partial junctional targeting of MBS because the protein localizes normally in epithelia lacking PATJ (Fig. S3 B). This is in line with the observation that both proteins localize differently during cellularization (Fig. 4 D).

To address the question whether the Patj-MBS interaction affects in vivo Myosin localization and/or phosphorylation, we segmentally overexpressed PATJ with engrailed::GAL4. Indeed, GFP-tagged Sqh (expressed under its endogenous promoter (Royou et al., 2002) or with a Polyubiquitin promoter) becomes strongly enriched at the junctional belt in the parasegment with PATJ-HA expression (Fig. 4 F and data not shown).

However, we were not able to detect a significant increase in Sqh phosphorylation upon segmental PATJ-overexpression (data not shown). Only upon the introduction of one mutant allele for *mbs*, phosphorylated Sqh is upregulated in stripes with PATJ overexpression (Fig. 4 G, control shown in Fig. S3 C), indicating that PATJ affects Myosin phosphorylation by inhibiting Myosin phosphatase.

This hypothesis is further supported by the fact that Myosin phosphorylation is significantly decreased in *PATJ*-mutant follicle cell clones (arrows in Fig. 4 H, mutant cells are marked by the absence of GFP).

Reduced MBS activity partly rescues *PATJ*-mutant phenotype

If loss of PATJ results in reduced inhibition of MBS and thus enhanced dephosphorylation of Myosin, reduction of MBS protein levels should counterbalance the *PATJ*-mutant phenotype. Therefore we analyzed the genetic interaction between *PATJ* and *mbs* and found that indeed the removal of one copy of *mbs* decreases the embryonic lethality of $PATJ^{\Delta 1}$ from 28 to 12% (Fig. 5 A). Furthermore, cuticle phenotypes (head defects, shortened cuticle) of $PATJ^{\Delta 1}$ mutant embryos are strongly decreased in a background heterozygous for a mutant *mbs* allele (Fig. 5 B). Interestingly, pupae homozygous mutant for $PATJ^{\Delta 1}$ and heterozygous for $mbs^{T541}$ start metamorphosis reflected by an elongation and remodeling of the wing disc (Fig. 5 C), a process which requires complex cell rearrangements and is thus highly dependent on Myosin dynamics (Pastor-Pareja et al., 2004), although in comparison to $PATJ^{\Delta 1}$ homozygous mutants, a similar percentage of flies survive until puparation. However, disc shape and morphology is not as elaborated as in pupae heterozygous mutant for *PATJ* (Fig. 5 C) or in wild type (data not shown). In contrast, wing discs in $PATJ^{\Delta 1}$ homozygous mutant animals appear normal in L3 larvae and in very early pupae but do not undergo morphological changes and finally disintegrate shortly after puparation (Fig. 5 C). In older $PATJ^{\Delta 1}$ / $PATJ^{\Delta 1}$, $mbs^{T541}$ mutant pupae, imaginal discs are also dissolved and pupal tissues become necrotic, indicating that removal of one allele of *mbs* does not fully rescue *PATJ* mutant flies, maybe because the correct balance between Myosin phosphorylation and dephosphorylation is not achieved upon removal of one intact *mbs* allele.

A further substantiation of our model came from the observation that the embryonic lethality upon overexpression of PATJ-GFP (Fig. S4 C) is decreased in flies overexpressing PATJ together with MBS (Fig. S4 C). Notably, overexpression of PATJ-GFP results in a

mislocalization of the overexpressed protein into the cytoplasma, whereas the junctional localization of DE-Cad and Myosin as well as apical-basal polarity is not affected (Fig. S4 A, PATJ-GFP expressed at lower levels under a ubiquitous promoter is shown as a control in B).

PATJ associates with Myosin in vivo

In order to investigate whether increased phosphorylation mediated by PATJ blocking Myosin phosphatase is the reason for Sqh accumulation in vivo (Fig. 4 F), we overexpressed PATJ in embryos expressing ubiquitously a non-phosphorylatable version of Sqh (Ubi::SqhAA-GFP). Surprisingly, SqhAA-GFP is similarly recruited to /stabilized at the apical junctions as its wild type counterpart (Fig. 6 A, control shown in Fig. S3 A), indicating that PATJ regulates Sqh not only by inhibiting Myosin phosphatase.

As increased phosphorylation of Sqh is obviously not the only mechanism to stabilize Myosin at the apical junction upon overexpression of PATJ, we elucidated the possibility that PATJ targets Myosin to the apical junctions by (direct or indirect) binding. Indeed, endogenous Zip coimmunoprecipitates with endogenous PATJ (Fig. 6 B). Due to the lack of an anti-Sqh antibody, which recognizes the endogenous protein in Western Blot, we verified that myc-tagged Sqh associates with PATJ-GFP in lysates from transfected S2R+ cells (Fig. 6 C). We further performed pulldown experiments with PATJ and Sqh expressed in and purified from *Escherichia coli* (*E. coli*) and found GST-PATJ to bind to MBP-Sqh in vitro (Fig. 6 D), suggesting that PATJ directly binds to Sqh and thereby might recruit Myosin to the apical junctions. In contrast, coimmunoprecipitation of PATJ with components of the AJ (DE-Cad and Arm) failed to confirm that PATJ associates with the core AJ (data not shown). These data indicate that PATJ forms a Cadherin-independent platform for Myosin to be activated and further locally inhibits Myosin phosphatase in order to enhance Myosin phosphorylation and thereby activity. This hypothesis is supported by the overlapping localization of PATJ and Zip (Fig. S2 E', arrows) as well as PATJ and phosphorylated Sqh (Fig. S2 F', arrows).

Finally we tested whether the amount of Myosin plays a role in *PATJ*-mutant phenotype: Increased Myosin levels upon overexpression of Sqh in a *PATJ*-mutant background decreased embryonic lethality as well as cuticle phenotypes (Fig. 6 E and data not shown). In contrast, overexpression of a non-phosphorylatable version of Sqh (SqhAA) does not affect *PATJ*-mutant phenotypes. This is compatible with our model that dephosphorylation of Sqh is enhanced in *PATJ* mutant embryos as an increment in (phosphorylatable) Sqh protein levels compensates in part for the increased dephosphorylation.

**Discussion**

Stabilization of AJ by an intact Actin-Myosin cytoskeleton is a crucial prerequisite for apical-basal polarity in epithelial cells (Ivanov et al., 2007; Shewan et al., 2005; Yamada and Nelson, 2007). However, to accomplish cell re-arrangements and thereby morphogenesis and cell migration, coordinated disassembly of AJ has to take place (Sandquist and Bement, 2010).

Many cell polarity regulators have been identified over the years to regulate AJ assembly and/or cell polarity in mammals and in the fly (Margolis and Borg, 2005). Whereas mammalian PATJ has been reported to regulate TJ formation and apical-basal polarity (Michel et al., 2005; Roh et al., 2002), up to now contradicting results obscured the role of *Drosophila* PATJ during development and in cell polarity (Djiane et al., 2005; Nam and Choi, 2006; Pielage et al., 2003; Richard et al., 2006; Tanentzapf et al., 2000).

In this study we demonstrate that in *Drosophila*, PATJ is only in part essential for embryonic development and does not regulate apical-basal polarity *per se*. Nonetheless *PATJ* is an essential gene and mutant flies die mostly in early pupal stages without proceeding in metamorphosis. These phenotypes are in line with a report which was published only recently, describing the effect of PATJ-alleles on apical-basal polarity and viability in flies (Zhou and Hong, 2012). In our study, we established a link between loss of PATJ and Myosin-dependent AJ stability: AJ with reduced E-Cadherin activity do not stably recruit Myosin and finally disintegrate when PATJ is absent. Our results indicate that PATJ can recruit Myosin to the apical junction belt by directly binding to Sqh and that PATJ further enhances Myosin activity through inhibition of Myosin phosphatase.

In the presence of intact AJ, PATJ seems to be dispensable for junction stability, cell polarity and most morphological rearrangements. However, a certain percentage of embryos show impaired germ band retraction and defects in the secretion of head cuticle, both processes with a high turnover and dynamic of the AJ. Similarly, *PATJ*-mutant imaginal discs do not undergo any morphological re-arrangements, indicating that PATJ plays a supporting role in the

modification of AJ in the embryo and an essential role during metamorphosis in the pupae. This is in line with our observation that in a background of reduced AJ stability, PATJ is essential for the stabilization of Myosin at the apical junctions and for the integrity of the AJ. Taken together we suggest here a model of PATJ recruiting Myosin to the apical junctions in redundancy with (an) other protein(s), which is/are likely to be associated with the AJ complex. Furthermore, PATJ enhances AJ stability and dynamics in tissues with intensive morphogenetic movements (e.g., imaginal discs during metamorphosis, head region in late embryonic development) by promoting Myosin phosphorylation through inhibition of Myosin phosphatase. The fact that a reduction in Myosin phosphatase activity not only rescues the embryonic lethality of $PATJ^{\Delta 1}$ to a far extent, but also results in a partial eversion of the imaginal discs, suggests that the lethality observed in *PATJ*-mutant flies is due to overactivation of Myosin phosphatase.

Our results are surprising with respect to previous studies in *Drosophila* and mammalian cells that postulate a crucial role for PATJ in apical-basal polarity and junction formation. One reason for these discrepancies in *Drosophila* might be that due to a lack of a clean *PATJ* allele, some studies have been carried out with deletions that are rescued by artificial constructs, which contained the N-terminus of PATJ (Nam and Choi, 2006; Pielage et al., 2003). Other reports used RNAi-mediated downregulation of PATJ, which bears the danger of off-targets and dose-dependent effects. However, PATJ might also play diverse roles in different cell types – in our study, we concentrated on embryonic and larval development and the embryonic epidermis as well as the follicular epithelium. Although most key players of cell polarity are present in the eye as well, polarity in photoreceptor cells differs from the epidermis in particular with respect to the role of Crb (reviewed by Bulgakova and Knust, 2009). Furthermore, although PATJ is well conserved during evolution, mammalian PATJ exhibits six additional PDZ domains (Roh et al., 2002), suggesting that it might be involved in other processes than the invertebrate protein.

Recently, mammalian PATJ was found to regulate apical constriction based on the AJ-associated Actin-Myosin belt by directly or indirectly recruiting the RhoGEF p114 to the apical junction (Nakajima and Tanoue, 2011). RhoGEFp114 activity is enhanced (in vitro) by Lulu2 (the mammalian homologue of *Drosophila* Yurt), which also concentrates at the AJ. RNAi-mediated downregulation of Lulu2 as well as of PATJ results in mislocalization of the junctional Actin-Myosin belt and impaired apical constriction. Furthermore, Shin et al. described PATJ to control cell migration in epithelial cells (Shin et al., 2007), which was supported by the observation that in migrating endothelial cells PATJ serves as a scaffold for Angiomotin and the RhoGEF Syx (Ernkvist et al., 2009). Unfortunately the authors did not test in this study whether cell migration is impaired in cells with decreased or abolished PATJ expression/activity and whether this is due to impaired RhoGEF activity. Although *Drosophila* PATJ has been found to indirectly associate with RhoGEF2 via slam (Wenzl et al., 2010), we did not detect any mislocalization of RhoGEF2 in *PATJ* mutant embryos that would substantiate the hypothesis that PATJ regulates Myosin dynamics via modulating RhoGEF2 (data not shown). Moreover, slam and RhoGEF2 are absent from mature AJ, further arguing against an implication of these two proteins in the PATJ-Myosin interaction described here (data not shown and Lecuit et al., 2002). Further studies are needed to determine whether the PATJ-mediated inhibition of Myosin dephosphorylation we described in this study also contributes to the migration and morphogenetic defects observed in mammalian cells.

One more indirect mechanism for PATJ regulating Myosin dynamics might be due to the fact that PATJ can directly bind the PDZ-domain of  (Nam and Choi, 2003), although the physiological relevance of this interaction needs to be further investigated in epithelial cells. Nonetheless, a physical link (via ) to Cdc42 thereby can be established, which might result in local modification of the Actin-cytoskeleton and AJ through Cdc42 activity (Samarin and Nusrat, 2009). Moreover, Crb itself and Sdt are also capable to bind  (and possibly indirectly Cdc42) in vitro (Hurd et al., 2003; Kempkens et al., 2006; Penkert et al., 2004; Wang et al.,

2004) – however, our data do not point at redundant functions of the Crb/Sdt complex and PATJ regarding the regulation of Actin-Myosin at the AJ (data not shown).

Under physiological conditions, PATJ seems to play only a subtle or redundant role in Myosin-dependent processes, as we did not observe any defects in cellularization, germ band extension or dorsal closure in *PATJ*-mutant embryos. Further analysis is required to clarify whether PATJ plays a role in these morphological rearrangements or whether its role is masked by other protein(s) that function in redundancy to PATJ.

Interestingly, there are at least two examples of AJ-associated proteins that have been described to play fundamental roles in vertebrate junction-assembly but do not show obvious phenotypes in *Drosophila*: Vinculin, an Actin-binding protein which stabilizes AJ and focal adhesions, essential in some mammalian tissues but dispensable in the fly (Alatortsev et al., 1997; Xu et al., 1998; Zemljic-Harpf et al., 2007). Similarly, p120-catenin modulates AJ assembly in mammalian cells (reviewed by Anastasiadis and Reynolds, 2000), but in *Drosophila p120-catenin* mutant alleles are viable and do not exhibit major AJ abnormalities or polarity defects (Myster et al., 2003). However, loss of p120-catenin strongly enhances *arm* and *shg* hypomorphic alleles, indicating that in cells with attenuated AJ, p120-catenin plays a crucial role in stabilizing the ZA, which is similar to the genetic interaction we observed between $shg^{R69}$ and $PATJ^{\Delta 1}$.

**Experimental Procedures**

Fly stocks and genetics

The *PATJ*$^{\Delta 1}$ allele was created as described by Hong *et al*. (Huang et al., 2008): In brief, a mini-white gene flanked by sequences homologous to the 3.5kbp upstream and the 3.5kbp downstream region of the genomic region encoding the PATJ open reading frame was linearized in females using heat-shocked induced Sce-I enzyme. Homologues recombination between the linearized cassette and the PATJ-genomic region took place in the female germline, resulting in progeny containing the mini-white gene instead of the region encoding the PATJ open reading frame.

The following mutant alleles were further used: *shg*$^{R69}$ (strong loss of function allele, Godt and Tepass, 1998), *mbs*$^{T541}$ (loss of function allele, Lee and Treisman, 2004). Two lines for Sqh-GFP expressed under its endogenous promoter were used (Royou et al., 2002). Identification of homo-/heterozygous mutant alleles was done using GFP- and RFP marked balancers.

*PATJ* germ line clones were generated with *PATJ*$^{\Delta 1}$ recombined with FRT2A using dominant female sterile technique (Chou et al., 1993): Thereby, only oocytes homozygous for the *PATJ*-mutant develop whereas heterozygous mutant oocytes as well as oocytes homozygous for the FRT2A-OvoD1 allele die early in oogenesis. These females were mated with males heterozygous for *PATJ*$^{\Delta 1}$ and homozygous mutant embryos were identified by absence of PATJ-staining in immunofluorescence. Ubi::PATJ-GFP, UASp::PATJ-HA, Ubi::Sqh-GFP, Ubi::SqhAA-GFP, UASp::Sqh-GFP and UASp::SqhAA-GFP transgenes were generated using phiC31-mediated germ line transformation, (Groth et al., 2004), attP40 and attP-VK00002 were used as landing sites.

The following GAL4 lines were used: da::GAL4 (#5460), en::GAL4 (#6356), arm::GAL4 (all obtained from Bloomington *Drosophila* Stock Center).

DNA and constructs

Cloning of the cDNA of wild type MBS, PATJ and Sqh into pENTR (Invitrogen) was performed using standard PCR on full length EST clones (*Drosophila* Genomics Resources Center, DGRC) as templates using the following primers:

MBS-F: 5'-CACCATGTCCTCGCTGGACG-3',

MBS-R: 5'- TTTACTTAATTTGCTAATTACTCTAA-3',

PATJ-F: 5'-CACCATGCACCTCAGCGCGGA-3',

PATJ-R: 5'-GTTCCGCCAGTCGGGAATCA-3',

Sqh-F: 5'-CACCATGTCATCCCGTAAGACCG-3',

Sqh-R: 5'-CTGCTCATCCTTGTCCTTG-3'.

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

SqhAA-F: AAGCGCGCCCAACGCGCCGCGGCCAATGTGTTCGCC

SqhAA-R: GGCGAACACATTGGCCGCGGCGCGTTGGGCGCGCTT

Constructs were recloned into destination vectors (PWG, UWG, Murphy lab, DGRC) using the gateway technology (Invitrogen).

Antibodies

Antibodies directed against *Drosophila* PATJ were raised by injection of a fusion protein of full length PATJ and GST into guinea pigs (Amsbio, Abingdon, UK).

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 guinea pig anti PATJ (this study) or 2 µl of the corresponding preimmune serum were added to cell

lysate corresponding to 500 µg total protein. Immune complexes were harvested using protein A-conjugated agarose (BioVision), washed five times in lysis buffer and boiled in 2x SDS sample buffer before SDS-PAGE and Western blot. For precipitation of PATJ-GFP from S2R+ cells, GFP-binder (ChromoTek, Planegg-Martinsried, Germany) was used.

Western blotting was done according to standard procedures. Primary antibodies used for Western blotting were as follows: guinea pig anti PATJ (1:2000, this study), rabbit anti Zip (1:2000, Liu et al., 2008), guinea pig anti pSqh (1:400, Zhang and Ward, 2011), mouse anti α-tubulin (12G10, 1:100, Developmental Studies Hybridoma Bank, DSHB), mouse anti Myc (9E10, 1:100, DSHB), rabbit anti GFP (#A11122, 1:1000, Life technology).

GST pull-down

Full length PATJ fused to GST was expressed in BL-21–competent bacterial cells and purified using glutathione beads (Macherey-Nagel). Full length Sqh and MBS fused to Maltose binding protein (MBP) was expressed accordingly and purified with Amylose-Resin (New England Biolabs). For PATJ-MBS pull-down experiments, 1µg MBP-MBS was incubated with equal amounts of either GST-PATJ or GST bound to glutathione beads in lysis buffer for 2 h at 4°C. After five washing steps in lysis buffer, beads were processed for Western blotting as described above. For PATJ-Sqh pull-down experiments, the same protocol was applied using MBP-Sqh, GST-PATJ and MBP alone as negative control. Amylose-Resin instead of glutathione beads were used to pull down MBP/MBP-Sqh. Rabbit anti GST (Sigma, 1:10000) and rabbit anti MBP (Sigma, 1:10000) was used.

Immunohistochemistry

Embryos were fixed in 4% formaldehyde, phosphate buffer pH 7.4 as described before (Krahn et al., 2009). Primary antibodies used for indirect immunofluorescence were as follows: Rabbit anti MBS (1:1000, Mizuno et al., 2002), guinea pig anti PATJ (1:500, this study), mouse anti Sdt (1:20, Berger et al., 2007), rabbit anti Baz (1:2000, Wodarz et al., 1999),

rabbit anti Zip (1:2000, Liu et al., 2008), mouse anti Sqh (1:1000, Zhang and Ward, 2011), guinea pig anti pSqh (1:100, Zhang and Ward, 2011), mouse anti Crb (Cq4, 1:50, DSHB), mouse anti Dlg (4F3, 1:50, DSHB), rat anti DE-Cad (DCAD2, 1:50, DSHB), mouse anti GFP (3E6, 1:1000, Life technologies), rat anti HA (3F10, 1:1000, Roche). 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 using either a 25x (NA 0.8, ZEISS) or a 63x (NA 1.2, ZEISS) water-objectives and ZEN2010 software. Images were processed using Adobe Photoshop.

Online supplemental material

Fig. S1 shows loss of PATJ in Western Blot, embryos and follicle cells. In Fig. S2 control stainings related to Fig. 3 are assembled. Fig. S3 demonstrates that MBS localizes normally in *PATJ*-mutant epithelia. Fig. S4 shows phenotypes of PATJ-GFP overexpression.
Video 1 and 2 show the embryonic development of a wild type embryo and a *PATJ*-mutant embryo, respectively.

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**Abbreviations used in this paper:** AJ, Adherens Junctions; aPKC, atypical protein kinase C; Arm, Armadillo; Baz, Bazooka; Crb, Crumbs; DE-Cad, *Drosophila* E-cadherin; Dlg, Discs Large; IP, immunoprecipitation; Lgl, Lethal (2) Giant Larvae; MBS, Myosin-Binding Subunit; MHC, Myosin Heavy Chain; MRLC, Myosin Regulatory Light Chain; MRLCK, Myosin Regulatory Light Chain Kinase; PATJ, Pals1-associated tight junction protein; Rok, Rho-associated Kinase; Sdt, Stardust; shg, shotgun; Sqh, Spaghetti squash; TJ, Tight Junctions; ZA, *Zonula Adherens*; Zip, Zipper.

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**Figure legends**

**Figure 1**. **Localization of PATJ during epithelial polarization.** (A-C) Endogenous PATJ localizes at the tip of the invaginating plasma membrane during cellularization, colocalizing with Sqh (D-E). (F) Upon gastrulation, PATJ is recruited to the apical AJ and localizes at the apical junctional region in mature epithelial cells (G). Scale bars = 5µm.

**Figure 2**. **PATJ is not essential for apical-basal polarity**. (A) Lethality of flies homozygous for *PATJ* $^{\Delta 1}$, data were averaged from three different experiments with 100 embryos each. *PATJ* $^{\Delta 1}$*/PATJ* $^{\Delta 1}$ represent embryos homozygous mutant for *PATJ* which still contain the maternal component, *PATJ* $^{\Delta 1}$ GLC are embryos derived from *PATJ* $^{\Delta 1}$ germ line clones, which lack maternal and zygotic PATJ expression. (B-G) Immunostainings of *PATJ*-mutant and wild type embryos. (B and C) Overview of wild type and mutant embryos, the head region is indicated by an arrow and the posterior end of the germ band is marked by an arrow head. Note that germ band retraction is not completed in the embryo homozygous for *PATJ* $^{\Delta 1}$ resulting in a posterior end at ca. 20% embryo length. This embryo also displays head defects. (D-G) Higher magnifications of wild type and *PATJ*-mutant embryos (derived from *PATJ* $^{\Delta 1}$ germ line clones) at stages 12/13 (shown is the mature epithelium of the embryonic epidermis), stained against DE-Cad/Dlg and Crb/Dlg, respectively. (H) Cuticle phenotypes of wild type embryos (left panel) and embryos homozygous mutant for *PATJ* $^{\Delta 1}$ (right panel). (I) Quantification of cuticle phenotypes from *PATJ* $^{\Delta 1}$ homozygous embryos. Cuticles were scored from three independent experiments with total numbers of embryos=174. (J) Follicle cell clones for *PATJ* $^{\Delta 1}$ showing loss of PATJ-staining and decreased protein levels of Sdt at the apical junction (arrows). *PATJ*-mutant clones are marked by the absence of GFP. Scale bars = 200µm in (B, C and H), 5µm in (D-G), 10µm in (J).

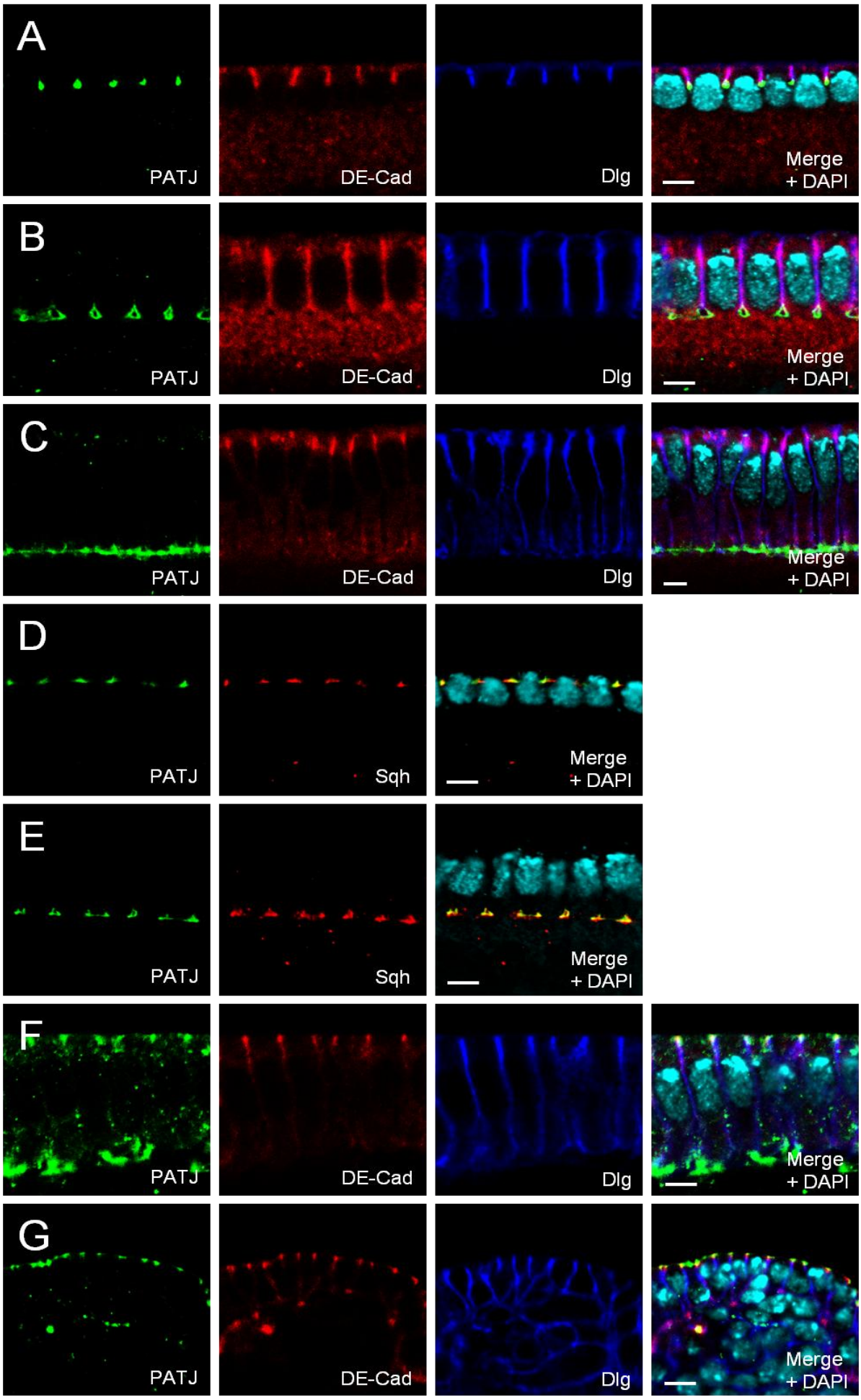
**Figure 3**. **PATJ supports weak AJ**. (A and C) Myosin heavy (Zip) and light (Sqh) chain accumulate at the apical junctional region in the embryonic epidermis, even in embryos expressing reduced levels of PATJ and DE-Cad (embryos heterozygous for *PATJ* $^{\Delta 1}$ and

*shgR69*). (B and D) Myosin is lost from AJ in embryos homozygous for *PATJ* $^{\Delta 1}$ and heterozygous for *shgR69*, although DE-Cad (B, D) as well as Arm (E) still accumulate at the ZA. (F-G) In later stages of embryos homozygous for *PATJ* $^{\Delta 1}$ and heterozygous for *shgR69*, DE-Cad and Baz mislocalize in cytosolic vesicles or in aggregates. Note that the epidermis appears multilayered and many cells start to round up, resulting in an unpolarized distribution of the lateral marker Dlg. (H) In the epidermis of these embryos, many cells undergo apoptosis, marked here by TUNEL labeling. (I) A reduction of DE-Cad protein level by introducing one mutant allele results in an increase of lethality and cuticle phenotypes in *PATJ* $^{\Delta 1}$ mutant embryos. Lethality data were averaged from three different experiments with 100 embryos each. Cuticles were scored from three independent experiments with total embryos 174 (*PATJ* $^{\Delta 1}$/ *PATJ* $^{\Delta 1}$) and 272 (*shgR69*/+; *PATJ* $^{\Delta 1}$/ *PATJ* $^{\Delta 1}$). Scale bars = 5μm in (AG), 200μm in H.

**Figure 4**. **PATJ enhances Myosin phosphorylation by inhibiting Myosin Phosphatase.** (A) Endogenous MBS coimmunoprecipitates with endogenous PATJ from embryonic lysates. The figure represents blots from different gels with 5% (PATJ-blot) and 95% of the IP loaded. (B) MBS-myc coimmunoprecipitates with PATJ-GFP from lysates of transfected S2R+ cells. (C) PATJ binds directly to MBS. GST-PATJ and MBP-MBS were expressed in *E.coli* and purified. GST alone served as negative control. Inputs are shown on coomassie-stained gel. (D-E) Localization of endogenous MBS during cellularization (D) and in mature epithelial cells of the epidermis (E, junctional MBS is marked by an arrow). (F) Overexpression of PATJ-HA in stripes using an engrailed::GAL4 driver line stabilizes/recruits Sqh-GFP in the embryonic epidermis. Sqh-GFP was expressed under its endogenous promoter (Royou et al., 2002). Here we used an insertion on the third chromosome, resulting in a rather low protein expression. Similar results were obtained using a ubiquitous promoter (Polyubiquitin, data not shown). (G) Segmental overexpression of PATJ-HA results in an increased phosphorylation of Sqh in embryos heterozygous for *mbs*$^{T541}$.

**Figure 5**. **PATJ and MBS interact genetically.** (A) Reduction of MBS can partly rescue the embryonic lethality of *PATJ* $^{\Delta 1}$. Lethality data were averaged from three different experiments with 100 embryos each. (B) Embryos heterozygous for *mbsT541* and homozygous for *PATJ* $^{\Delta 1}$ show less cuticle defects than embryos homozygous for *PATJ* $^{\Delta 1}$. Cuticles were scored from three different experiments with total numbers of embryos 174 (*PATJ* $^{\Delta 1}$/ *PATJ*$^{\Delta 1}$) and 95 (*PATJ* $^{\Delta 1}$/*mbsT541,PATJ* $^{\Delta 1}$). (C) Pupae homozygous for *PATJ* $^{\Delta 1}$ and *mbs*$^{T541}$ start metamorphosis in the imaginal discs. Scale bar in (C) = 100μm.

**Figure 6**. **PATJ associates with Myosin in vitro and in vivo**. (A) Segmental overexpression of PATJ-HA stabilizes a Sqh protein, which cannot be phosphorylated, at the AJ (SqhAAGFP). (B) Endogenous Zip can be co-purified together with PATJ from embryonic lysates. Both blots are from the same gel. (C) Coimmunoprecipitation of PATJ-GFP and Sqh-myc from transfected S2R+ cells. (D) GST-PATJ directly associates with MBP-Sqh in a MBPpulldown assay. (E) Overexpression of wild type Sqh but not of a phosphorylation-deficient version (SqhAA) can partly rescue PATJ-mutant embryonic lethality. Lethality data were averaged from three different experiments with 100 embryos each. Scale bar in (A) = 10μm.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PATJ
DE-Cad
Dlg
Merge
+ DAPI
B
PATJ
DE-Cad
Dlg
Merge
+ DAPI
C
PATJ
DE-Cad
Dlg
Merge
+ DAPI
D
PATJ
Sqh
Merge
+ DAPI
E
PATJ
Sqh
Merge
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G
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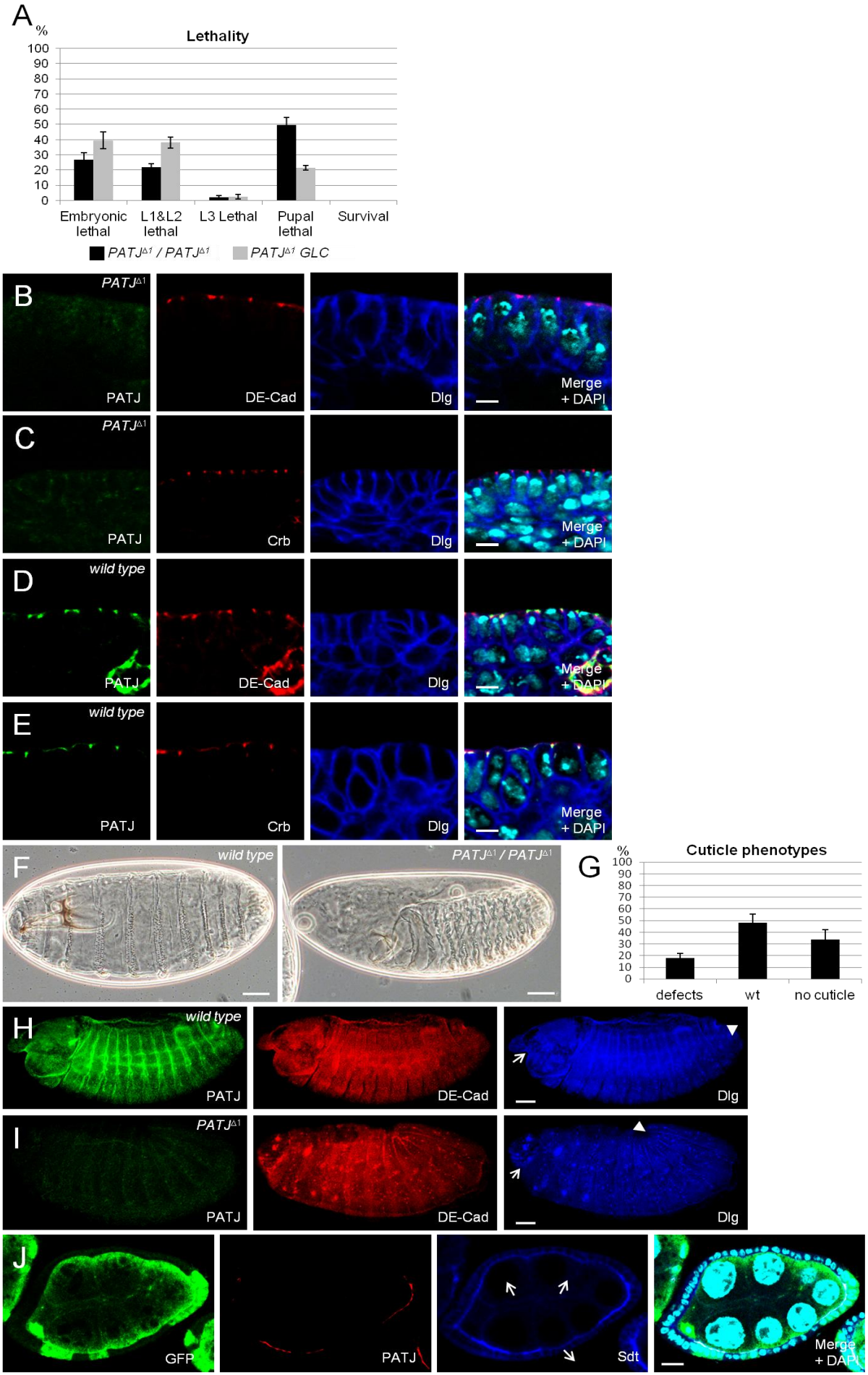
**Figure 1**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Embryonic lethal
L1&L2 lethal
L3 Lethal
Pupal lethal
Survival
PATJΔ1 / PATJΔ1
PATJΔ1 GLC
B
PATJΔ1
PATJ
DE-Cad
Dlg
Merge + DAPI
C
PATJΔ1
PATJ
Crb
Dlg
Merge + DAPI
D
wild type
PATJ
DE-Cad
Dlg
Merge + DAPI
E
wild type
PATJ
Crb
Dlg
Merge + DAPI
F
wild type
PATJΔ1 / PATJΔ1
G
Cuticle phenotypes
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defects
wt
no cuticle
H
wild type
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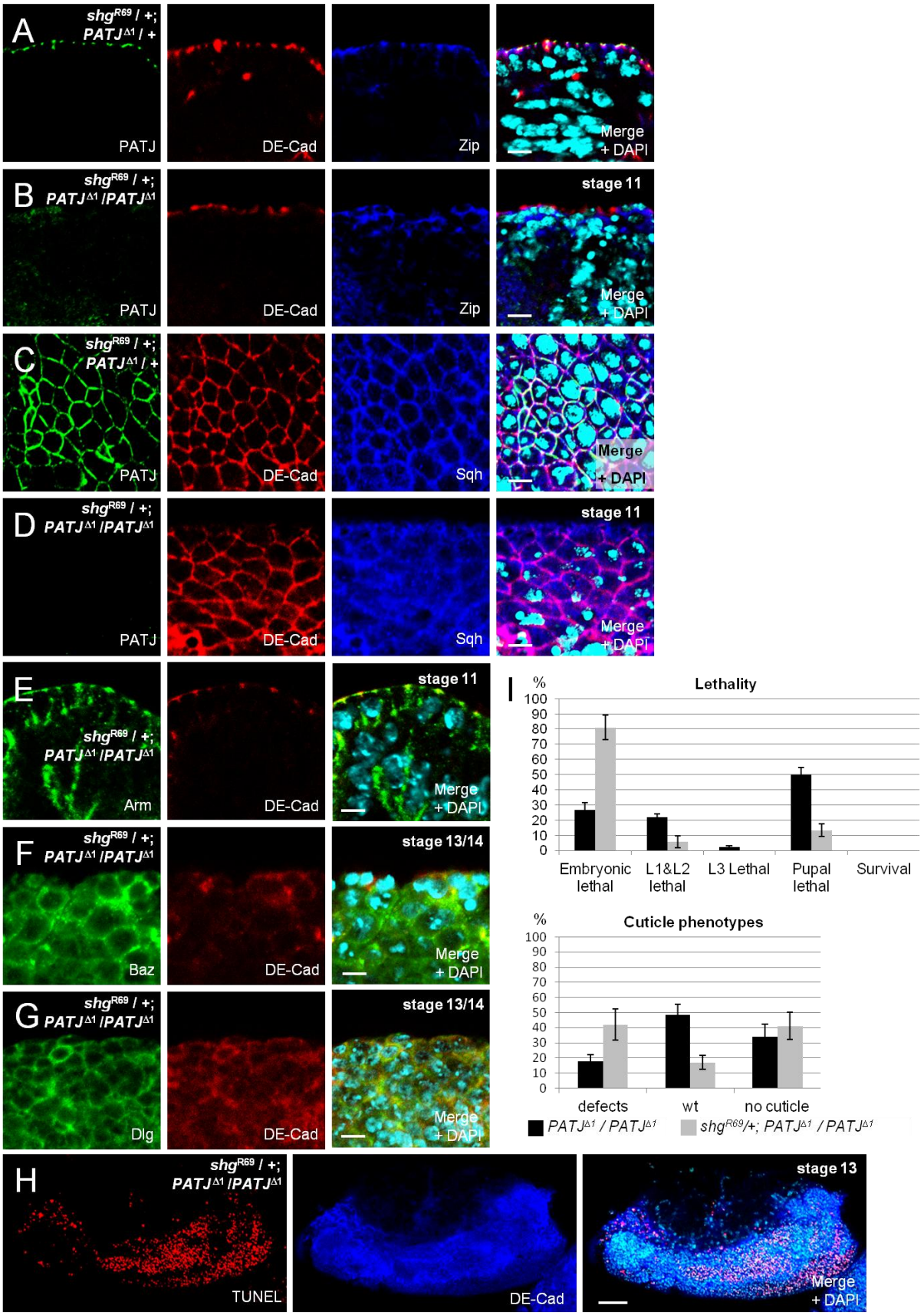
PATJ
DE-Cad
Dlg
J
GFP
PATJ
Sdt
Merge + DAPI

**Figure 2**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shgR69 / +;
PATJΔ1 / +
PATJ
DE-Cad
Zip
Merge
+ DAPI
B
shgR69 / +;
PATJΔ1 /PATJΔ1
stage 11
C
Sqh
D
E
Arm
F
Baz
stage 13/14
G
Dlg
H
TUNEL
stage 13
I
Lethality
%
Embryonic lethal
L1&L2 lethal
L3 Lethal
Pupal lethal
Survival
Cuticle phenotypes
defects
wt
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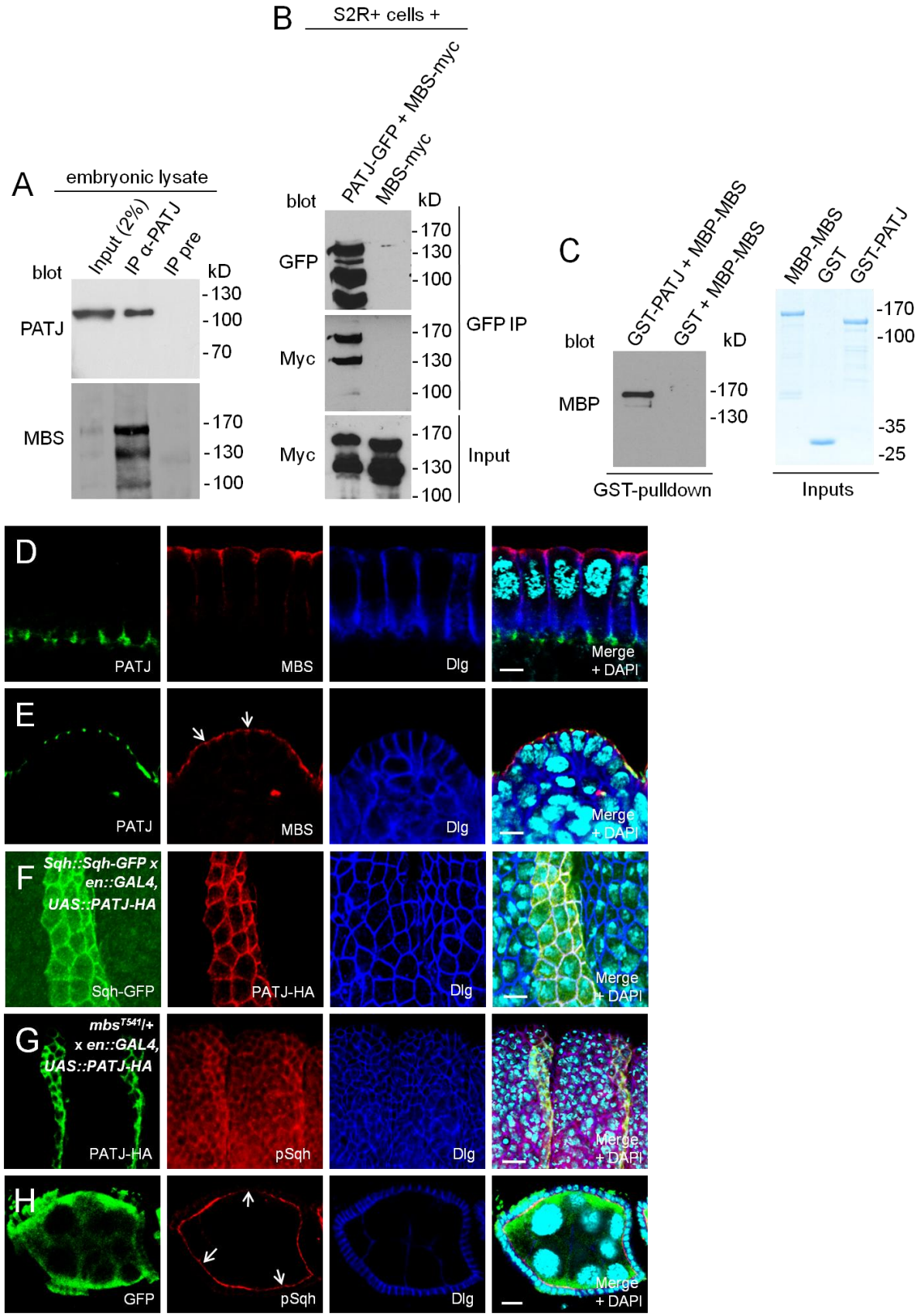
PATJΔ1 / PATJΔ1
shgR69/+; PATJΔ1 / PATJΔ1

**Figure 3**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Input (2%)
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IP pre
blot
kD
PATJ
-130
-100
-70
MBS
-170
-130
-100
B
S2R+ cells +
PATJ-GFP + MBS-myc
MBS-myc
blot
kD
GFP
-170
-130
-100
Myc
-170
-130
-100
GFP IP
Myc
-170
-130
-100
Input
C
GST-PATJ + MBP-MBS
GST + MBP-MBS
blot
kD
MBP
-170
-130
GST-pulldown
MBP-MBS
GST
GST-PATJ
-170
-100
-35
-25
Inputs
D
PATJ
MBS
Dlg
Merge
+ DAPI
E
PATJ
MBS
Dlg
Merge
+ DAPI
F
Sqh::Sqh-GFP x
en::GAL4,
UAS::PATJ-HA
Sqh-GFP
PATJ-HA
Dlg
Merge
+ DAPI
G
mbsT541/+
x en::GAL4,
UAS::PATJ-HA
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pSqh
Dlg
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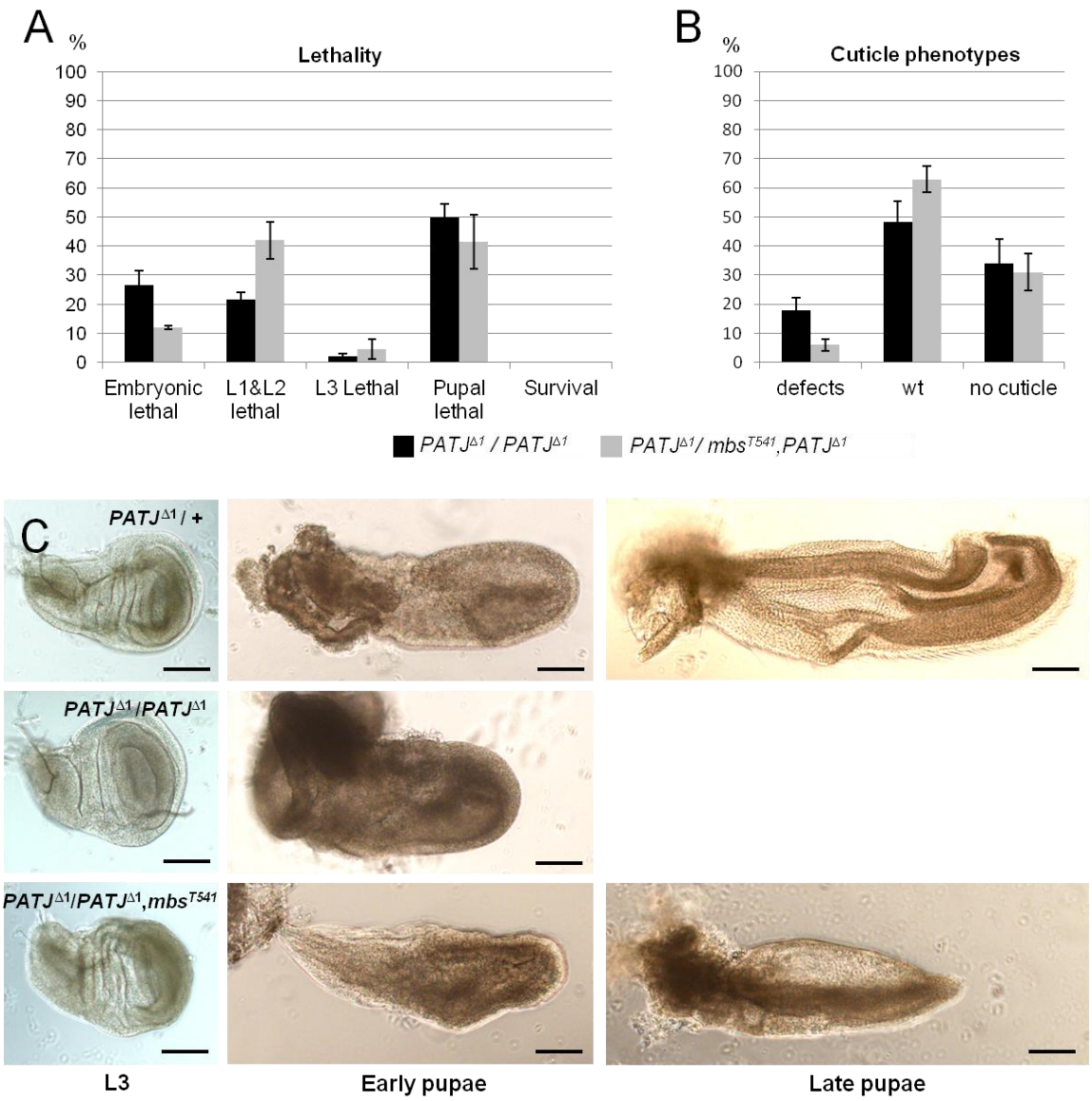
H
GFP
pSqh
Dlg
Merge
+ DAPI

**Figure 4**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Lethality
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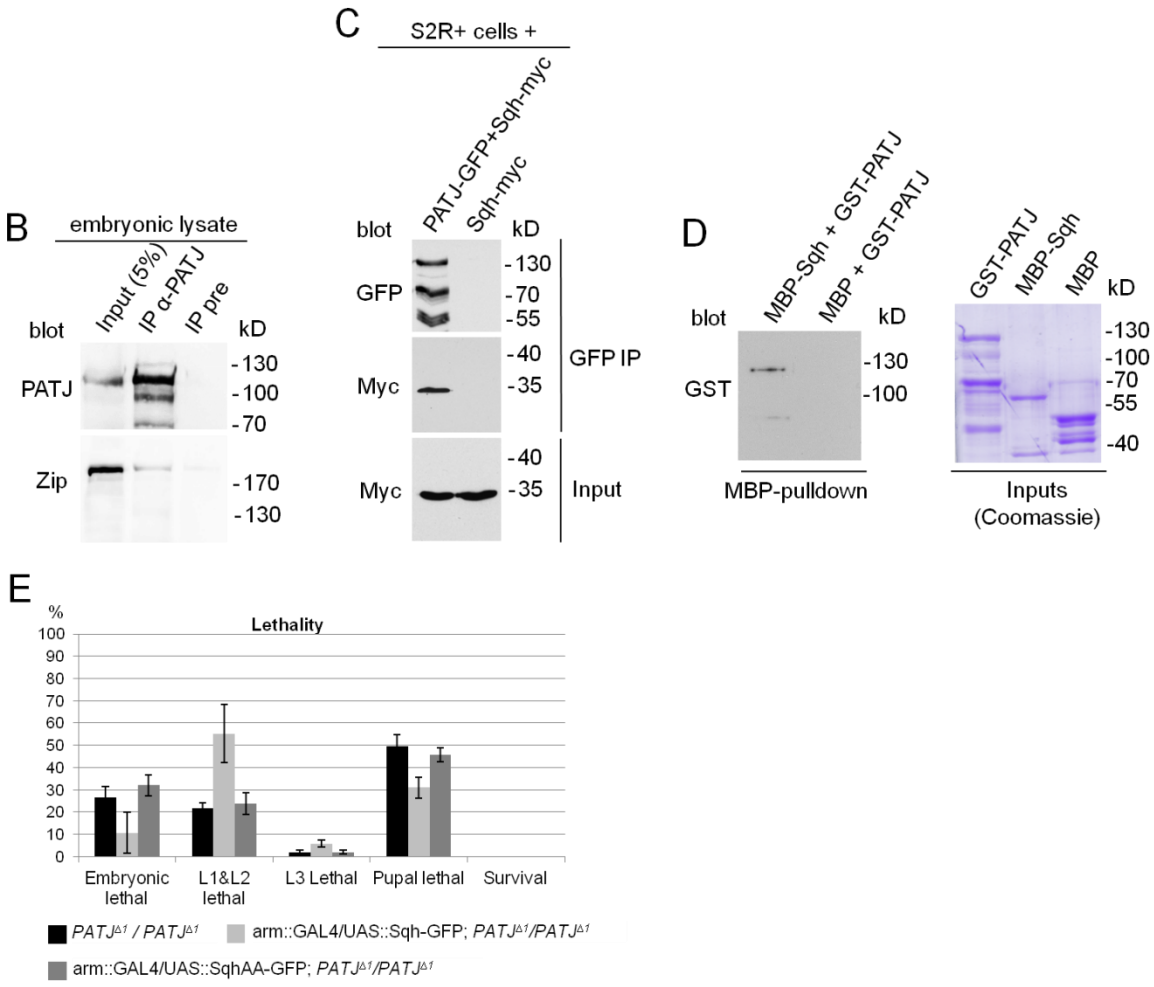
Embryonic lethal
L1&L2 lethal
L3 Lethal
Pupal lethal
Survival
B
%
Cuticle phenotypes
100
90
80
70
60
50
40
30
20
10
0
defects
wt
no cuticle
PATJΔ1 / PATJΔ1
PATJΔ1/ mbsT541,PATJΔ1
C
PATJΔ1 / +
PATJΔ1/PATJΔ1
PATJΔ1/PATJΔ1,mbsT541
L3
Early pupae
Late pupae

**Figure 5**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embryonic lysate
Input (5%)
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IP pre
blot
kD
PATJ
-130
-100
-70
Zip
-170
-130
C
S2R+ cells +
PATJ-GFP+Sqh-myc
Sqh-myc
blot
kD
GFP
-130
-70
-55
Myc
-40
-35
GFP IP
Myc
-40
-35
Input
D
MBP-Sqh + GST-PATJ
MBP + GST-PATJ
blot
kD
GST
-130
-100
MBP-pulldown
GST-PATJ
MBP-Sqh
MBP
kD
-130
-100
-70
-55
-40
Inputs
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Lethality
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90
80
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0
Embryonic lethal
L1&L2 lethal
L3 Lethal
Pupal lethal
Survival
PATJΔ1 / PATJΔ1
arm::GAL4/UAS::Sqh-GFP; PATJΔ1/PATJΔ1
arm::GAL4/UAS::SqhAA-GFP; PATJΔ1/PATJΔ1

**Figure 6**

**Supplemental Figure Legends**

Figure S1. **PATJ-mutant embryos and follicle cells**. This figure is related to Fig. 2. (A) Western blot showing that PATJ protein expression is not detectable in embryos, which are maternal and zygotic mutant for *PATJ・1* but is still present in heterozygous embryos. Alpha-tubulin was used as loading control. (B) Embryos homozygous mutant for *PATJ* show only moderate apoptosis in the epidermis of late stage embryos. TUNEL-assay was used to identify apoptotic cells. (C) Follicle cell clones showing decreased protein levels of Crb at the apical junction (arrows). *PATJ*-mutant clones are marked by the absence of GFP. Scale bars = 200μm in (B), 10μm in (C).

Figure S2. **Localization of Arm, Baz and Dlg in PATJ-mutant embryos**. This figure is related to Fig. 3. Embryos homozygous for $PATJ^{\Delta 1}$ were stained against Arm (A), Baz (B), Dlg (C) and Zip (D). Localization of these proteins is indistinguishable from wild-type epithelial cells (Fig. 2 D-E and data not shown). (E) and (F), high magnifications of epithelial cells showing a partly overlapping localization of PATJ and DE-Cad (E and F) and PATJ and Zip (E') or pSqh (F') respectively. Cells are the same in (E) and (E') as well as in (F) and (F'). (G) PATJ and Baz are mislocalized to the cytoplasma in embryos homozygous mutant for $shg^{R69}$. Scale bars = 5μm.

Figure S3. **Localization of non-phosphorylatable GFP-Sqh in wild type embryonic epidermis**. This figure is related to Fig. 4. (A) Flies expressing SqhAA under a ubiquitous promoter were crossed with the driver line engrailed::GAL4 and stained against GFP, Patj and Dlg. Note that there is no accumulation of Sqh-GFP at the junctions in the absence of PATJ overexpression. (B) Localization of MBS in *PATJ* mutant epithelial cells is indistinguishable from wild type epithelia (junctional MBS is marked by an arrow). Scale bar in (A) = 10μm, in (B) = 5μm.

Figure S4. **Overexpression phenotypes of PATJ**. This figure is related to Fig. 5. (A) Overexpression of PATJ-GFP results in a mostly cytosolic protein localization, whereas Myosin and DE-Cad accumulate normal at the apical junctions. Daughterless(dag)::GAL4 was used to overexpress PATJ-GFP. (B) Moderate expression of PATJ-GFP by a ubiquitous promoter (Polyubiquitin) results in a physiological protein localization and functional protein (data not shown). (C) Overexpression of PATJ-GFP by dag::GAL4 results in an increased embryonic lethality which can be to some extent rescued by concomitant overexpression of MBS-myc. Scale bars = 5µm.

Video 1. **Life imaging of a wild type *Drosophila* embryo expressing DE-Cad-GFP**. This video is related to Fig. 2 and is shown at 10 frames/s. Frames were recorded every 6 minutes and each picture is a projection of a stack of four images to prevent out-of-focus slipping of the epidermis. . Anterior is in the lower left corner. Time in minutes is indicated. The video starts shortly after cellularization, showing the normal embryonic development of a representive wild type embryo with germ band elongation, segmentation, germ band retraction and finally dorsal closure and head development.

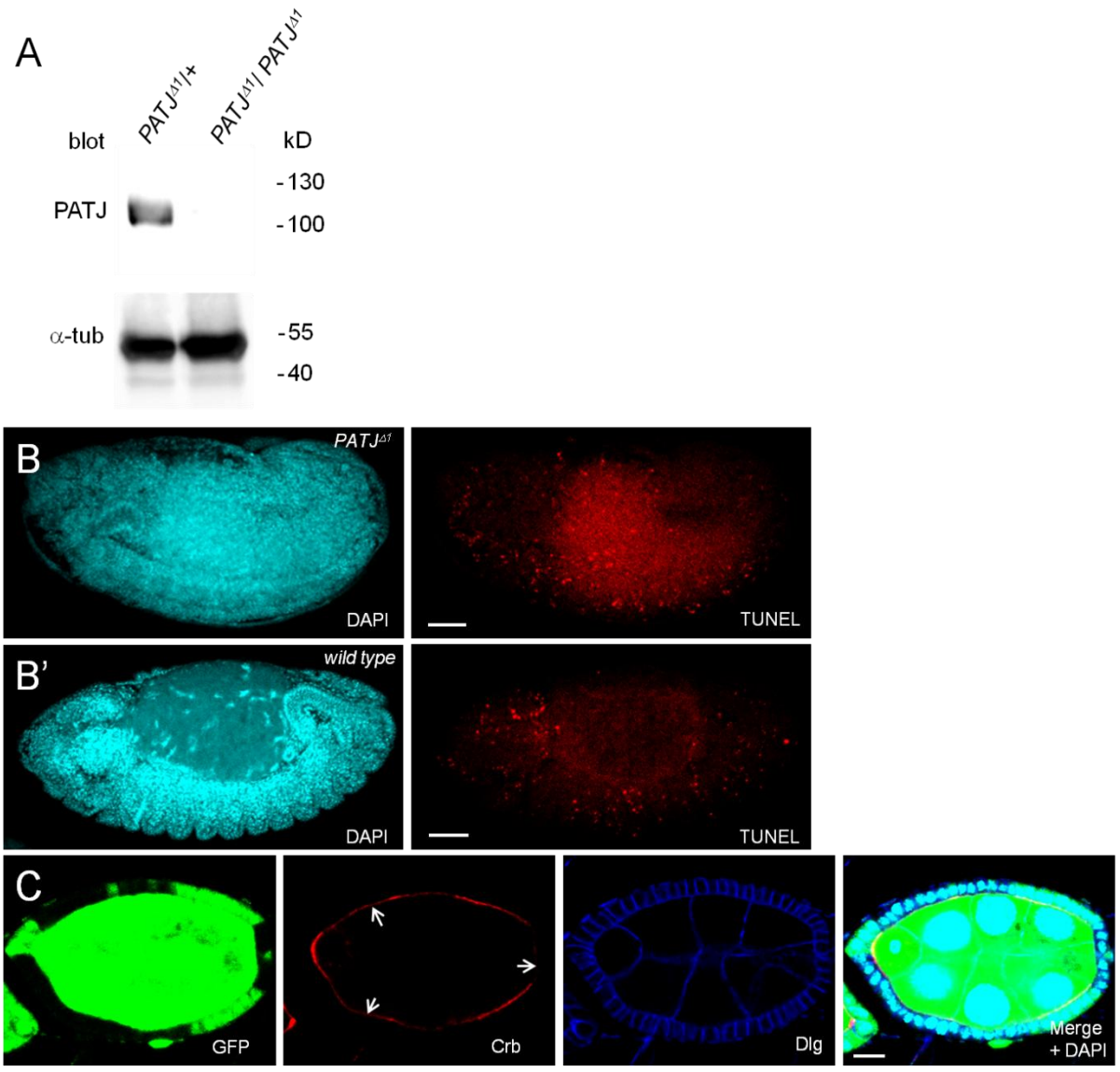
Link to Video 1: http://rup-movie.glencoesoftware.com/video/10.1083/jcb.201206064/video-1

Video 2. **Life imaging of a *PATJ*-mutant *Drosophila* embryo expressing DE-Cad-GFP**. This video is related to Fig. 2 and recorded as Video 1. Anterior is in the lower left corner. The embryo shown is derived from $PATJ^{\Delta 1}$ germ line clones, lacking the maternal and zygotic expression of PATJ.

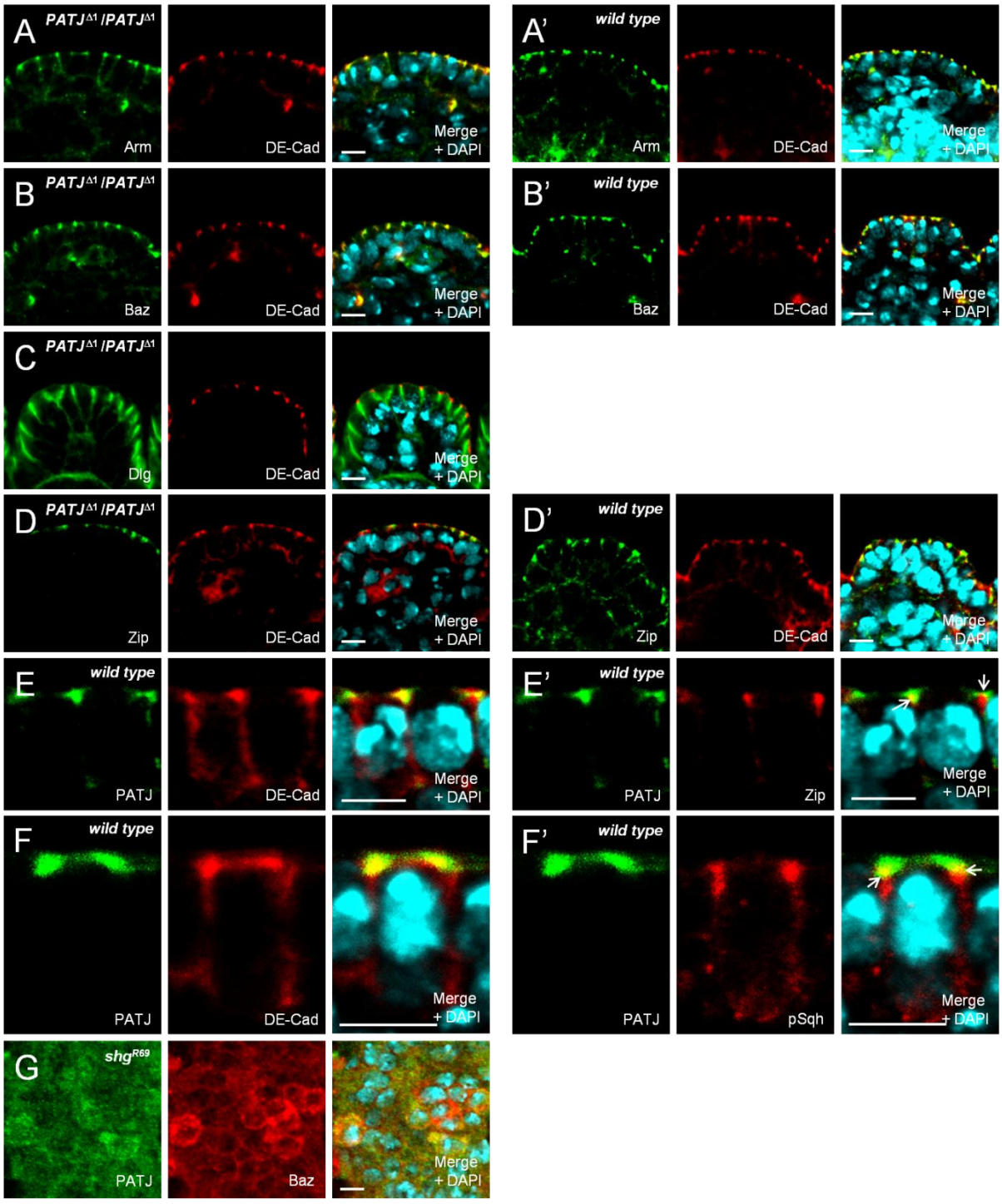
The video starts shortly after cellularization, showing a rather normal germ band elongation and segmentation but an incomplete germ band retraction with the posterior end of the germ band ending at ca. 20% embryonic length instead of 0% as in the wild type embryos (c.p. video 1). Out of 39 *PATJ*-mutant embryos investigated, 8 exhibited defects in germ band

retraction and head development, 5 died early in development and the rest developed normally.

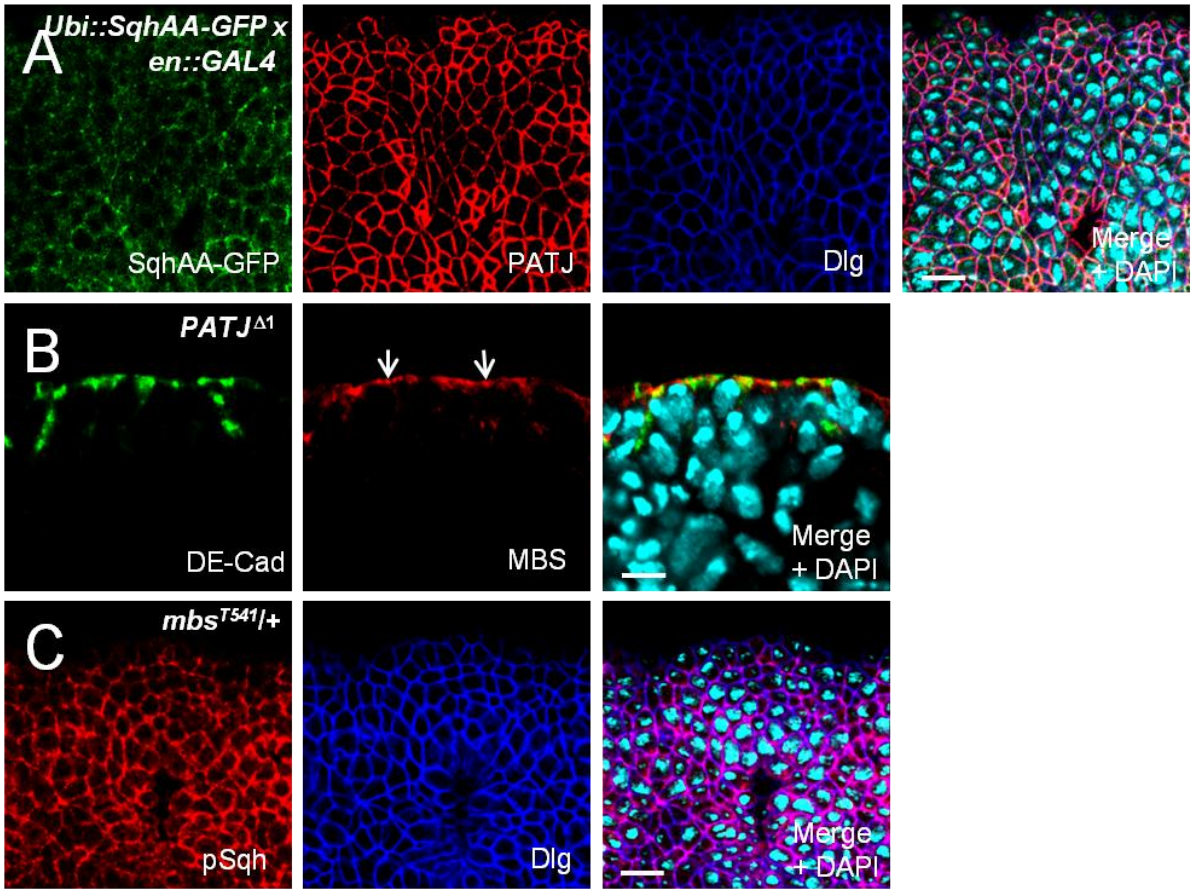
Link to Video 2: http://rup-movie.glencoesoftware.com/video/10.1083/jcb.201206064/video-2



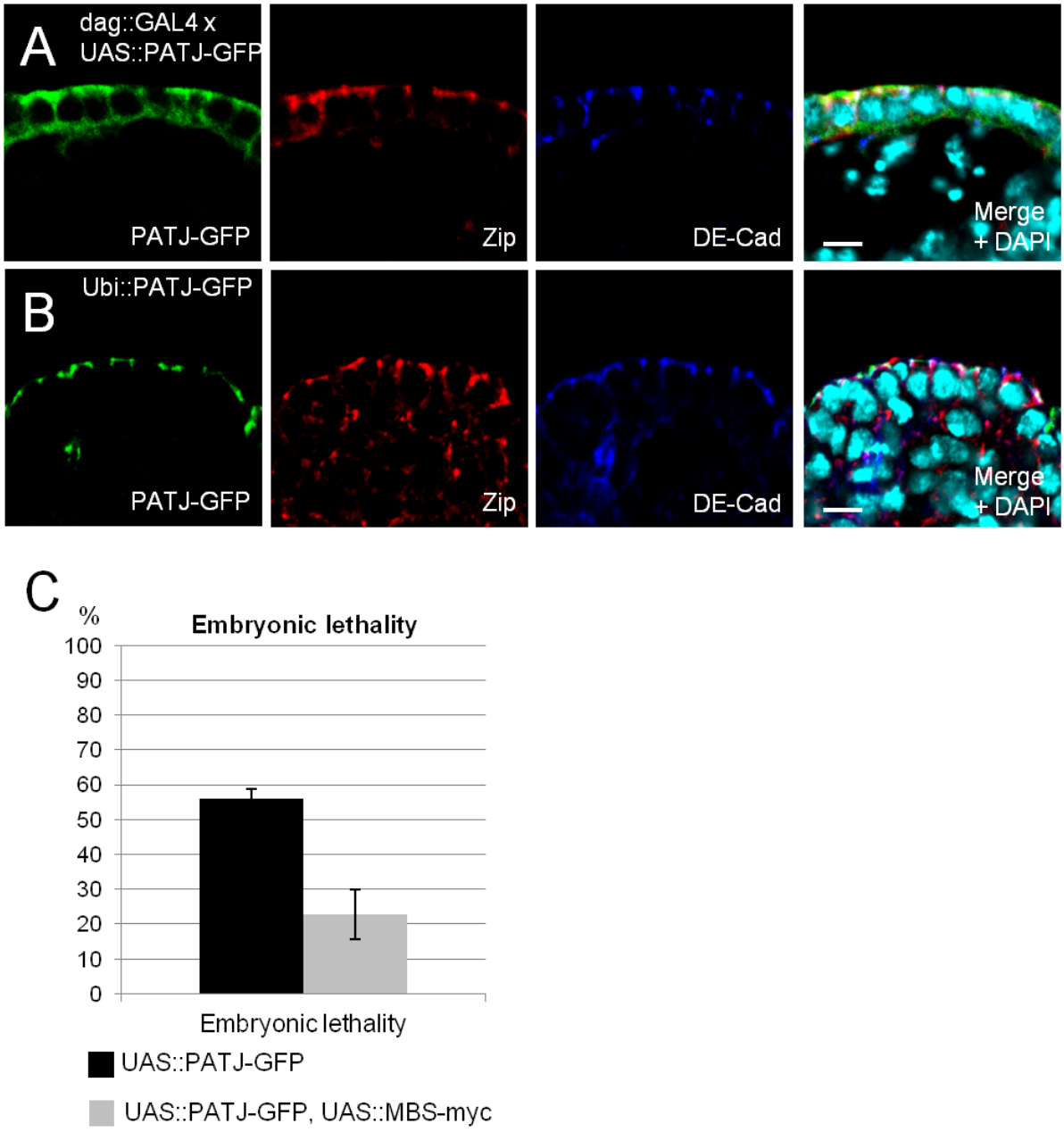

**Supplementary Figure 1**




**Supplementary Figure 2**




**Supplementary Figure 3**




**Supplementary Figure 4**

## 3.3 PAR-6 regulates apical-basal polarity in epithelia by preventing degradation of Sdt/Pals1

In this part of the project, we aimed to decipher the role of PAR-6 in regulating the Crb-Sdt-PATJ complex. Using immunofluorescence experiments we show that in *PAR-6* mutant embryonic and follicular epithelia members of the Crb complex, Crb and PATJ are mislocalized while Sdt is totally absent. Furthermore, we confirmed by Western Blot analysis that Sdt is degraded upon loss of PAR-6 in PAR-6 mutant embryos.

Although postulated from results of in vitro and overexpression studies in mammalian cells, we were not able to detect a robust interaction of Sdt-PAR-6 or Crb-PAR-6 in co-immunoprecipitation assays using endogenous proteins in embryonic lysates.

In a previous screen, PAR-6 has been described to interact with Rpn13, a proteasomal receptor. Strikingly, downregulation of a proteasomal core component (Rpn2) or Rpn13 in PAR-6 mutant cells rescues degradation of Sdt.

**Arnab Sen** and Michael P. Krahn

Author contributions to work:

**Arnab Sen:** all experiments, partly writing of the manuscript.

Michael P. Krahn: Editing of the manuscript.

**Status: Manuscript in preparation** (Estimated submission: end of June 2014)

# PAR-6 regulates apical-basal polarity in epithelia by preventing degradation of Sdt/Pals1

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**Cell polarity is one of the key prerequisites for the establishment of multicellular organisms. The PDZ- and PB1-domain containing protein PAR-6 associates with PAR-3, aPKC and Cdc42 in a quaternary complex (PAR-aPKC complex), modulating the kinase activity of aPKC and activating Cdc42, thus controlling cell polarity in various tissues and organisms(Suzuki and Ohno, 2006). Furthermore PAR-6 has been described to regulate the positioning of a second apical complex, consisting of Crumbs and Stardust/Pals1 (Hurd et al., 2003; Kempkens et al., 2006; Penkert et al., 2004; Wang et al., 2004). However, in *Drosophila* epithelial cells we found no robust association of PAR-6 with Crb/Sdt under endogenous conditions. Here we demonstrate that instead of a direct binding, PAR-6 modulates the stability of the Crb/Sdt complex. In *PAR-6*-mutant cells, Stardust is degraded, resulting in an intracellular mislocalization of Crumbs and subsequent disturbance of apical-basal polarity. These defects are independent of aPKC or Cdc42 activity. In contrast PAR-6 directly binds the proteasomal receptor Rpn13, thereby prevent the proteasomal degradation of Sdt. Downregulation of Rpn13 or integral components of the proteasome in *PAR-6*-mutant epithelial cells restores Crb/Sdt accumulation at the apical junctions. These results show**

**that instead of direct association with the Crb/Sdt complex, PAR-6 regulates its stability indirectly via selectively inhibiting the degradation of Sdt. Proteasomal inhibition (in contrast to proteasomal targeting) is a new mechanism for the establishment and maintenance of apical-basal polarity in epithelial cells, raising the questions whether it applies for other contexts of polarity, too.**

In *Drosophila* and mammalian epithelial cells, the PAR-complex defines together with the Crb-complex the apical plasmamembrane domain and regulates the formation of Adherens Junctions (AJ) and Tight-Junction (TJ) in vertebrates.

Several studies demonstrate direct and indirect interactions between these two complexes (Hurd et al., 2003; Kempkens et al., 2006; Krahn et al., 2010; Penkert et al., 2004; Wang et al., 2004). In *PAR-6*-mutant *Drosophila* epithelia, Crb is mislocalized (Kempkens et al., 2006) and PAR-6 supports Pals1-dependent TJ formation (Hurd 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 (a multiple PDZ-domain protein functioning downstream of Sdt) are mislocalized whereas staining of Sdt is almost absent (Fig. 1a-c and data not shown, note that cell morphology is strongly impaired upon loss of PAR-6). Notably, Bazooka (Baz, the PAR-3 homologue in *Drosophila*) still accumulates at distinct spots at the cell-cell contacts (Fig. 1a-c).

We previously found that Baz directly recruits Sdt to the apical junctions before the onset of Crb expression in the embryonic epidermis. Thus we tested whether PAR-6 supports the stability of a Baz-Sdt or Crb-Sdt complex by joining these complexes.

In contrast to previous studies which used recombinant proteins or protein overexpressed in cell culture experiments (Hurd et al., 2003; Kempkens et al., 2006; Penkert et al., 2004; Wang et al., 2004), we were not able to detect a physical association of PAR-6 with Crb or Sdt under endogenous conditions in embryonic lysates (Fig. 1d and e). Thus it is unlikely that PAR-6

controls the assembly of the Crb-Sdt complex in embryonic epidermis by direct interactions with its components.

As phosphorylation of Baz by aPKC results in a disassembly of the Baz-Sdt complex (Krahn et al., 2010), we next tested whether loss of PAR-6 results in a disturbed activity of aPKC. Several reports demonstrate that PAR-6 activates (Graybill et al., 2012; Yamanaka et al., 2001) or inhibits (Atwood et al., 2007) aPKC kinase activity. We found in *Drosophila* Schneider R+ cells (S2R+, Fig. 1f) that loss of PAR-6 abolished aPKC autophosphorylation, reflecting a decrease in its kinase activity. Notably, protein expression of aPKC was decreased, too, indicating that PAR-6 might either enhance expression of aPKC or stabilize this protein. However, expression of a constitutively active version of aPKC (Sotillos et al., 2004) in *PAR-6*-mutant follicle cells does not rescue Sdt/Crb localization, nor does expression of a dominant-negative aPKC in the same background (Fig. 1g and data not shown). Moreover, loss of aPKC in the embryonic epidermis or in follicle cells does not result in mislocalized Sdt, Crb or PATJ although cell morphology and apical-basal polarity is strongly impaired (Fig. 1h and data not shown). Finally expression of a mutant variant of Baz ($Baz_{S980A}$) which cannot be phosphorylated by aPKC and fails to disassemble from Sdt (Krahn et al., 2010) does not result in restored Sdt or Crb localization in *PAR-6*-mutant follicle cells (data not shown). These data indicate that PAR-6 controls the localization of Sdt and Crb independently of aPKC activity.

Western Blot analysis revealed that whereas Crb and PATJ protein expression is comparable to wild type in PAR-6 mutant cells, Sdt is almost entirely absent in embryos derived from *PAR-6* germ line clones (Fig. 2a). Thus PAR-6 controls selectively the stability of Sdt but not of Crb or PATJ (Fig. 2a). To investigate whether degradation of Sdt is mediated by the proteasome, we inhibited proteasomal degradation in *PAR-6*-mutant follicle cells by RNA-interference (RNAi)-mediated downregulation of a core proteasomal subunit (Rpn2). Indeed, reduction of Rpn2 results in a significant stabilization of Sdt in PAR-6 mutant follicle cells (Fig. 2b). Sdt as well as Crb colocalized with Baz at the apical cell-cell contacts (Fig. 2b,

arrow and data not shown), although the staining of all three proteins was much weaker compared to control cells. Furthermore, cell morphology was still severely disturbed in *PAR-6* mutant cells expressing Rpn2-RNAi, indicating that apart from its effect on Sdt stability, PAR-6 accomplishes other functions in epithelial polarity.

Mammalian PAR-6 can be activated by TGFβ or Wnt-signalling (via phosphorylated Dishevelled) resulting in binding of PAR-6 to the E3-ubiquitin ligase Smurf1, which subsequently leads to a local degradation of RhoA during cell migration or epithelial-to-mesenchymal transition or of the planar cell polarity regulator Prickle1(Narimatsu et al., 2009; Ozdamar et al., 2005). However, RNAi-mediated downregulation of neither Smurf1 (lack in *Drosophila*) nor of Dishevelled rescues the degradation of Sdt in *PAR-6*-mutant follicle cells (Fig. 2c and data not shown).

A second link between PAR-6 and the proteasome was established in a screen for direct interaction partners of PDZ-domain containing proteins in *C. elegans* (Lenfant et al., 2010). Here, the proteasomal receptor Rpn13 was found to interact with the PDZ-domain of PAR-6 in a Yeast-2-Hybrid assay. Rpn13 (also named Adrm1) links the proteasome-associated deubiquitinating enzyme UCH37 to the 26S proteasomes and thus functions as a receptor for ubiquitinylated proteins, enhancing their degradation (Hamazaki et al., 2006; Jorgensen et al., 2006; Qiu et al., 2006). In contrast to core components of the proteasome, loss of Rpn13 in yeast or mammalian cells had no or only subtle effects on overall protein polyubiquitination and –degradation, indicating that it might rather function in the degradation of a certain subset of proteins (Hamazaki et al., 2006; Jorgensen et al., 2006; Qiu et al., 2006). Notably, Rpn13 mRNA levels are upregulated in metastatic cells of human breast cancer cell lines (Simins et al., 1999).

Thus PAR-6 might modulate the degradation of Sdt by binding Rpn13 and thereby preventing Rpn13 from linking Sdt to the proteasome and subsequent degradation. This hypothesis is supported by the observation that RNAi-mediated downregulation of Rpn13 in PAR6-mutant

follicle cells (similar to Rpn2-RNAi) results in a stabilization of Sdt and an accumulation of Crb/Sdt at cell-cell contacts, where Baz is localized (Fig. 3a).

Taken together our data indicate that PAR-6 controls the function of Crb in epithelial cells by preventing proteasomal degradation of Sdt, which is turn stabilizes Crb at the apical cell-cell contacts. This process is selectively for Sdt as other polarity proteins (e.g. Crb or PATJ) are not or only slightly degraded upon loss of PAR-6. Thus we have demonstrated a new mechanism of PAR-6 in the establishment of apical-basal polarity involving inhibition instead of induction of proteasomal degradation.

**METHODS SUMMARY**

Germ line clones of a PAR-6 null allele (*PAR-6*$^{\Delta 22}$) and aPKC null allele (*aPKC*$^{k06403}$) were generated using dominant female sterile technique. RNAi was expressed in *PAR-6*$^{\Delta 22}$ mutant follicle cell clones using Actin::GAL4 and a FRT19A, tub::GAL80 allele.

DsRNA experiments in S2R+ cells were carried out as described before (Krahn et al., 2009), using 20µg/ml dsRNA. Immunoprecipitation, Western Blotting and staining of embryos and follicle cells was carried out as described before (Sen et al., 2012) using the following antibodies: guinea pig anti PAR-6 (1:500, Kim et al., 2009), guinea pig anti PATJ (1:1000, Sen et al., 2012), mouse anti Sdt (1:20, Berger et al., 2007), rabbit anti Baz (1:2000, Wodarz et al., 1999), rabbit anti pS980 Baz (1:200, Krahn et al., 2009), rabbit anti aPKC (aPKCξ, 1:500, Santa Cruz sc-216). Images were taken on a Zeiss LSM 710 Meta confocal microscope and processed using Adobe Photoshop.

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**Figure legends**

**Figure 1 PAR-6 controls localization of the Crb-Sdt-PATJ complex. A, B,** PATJ is mislocalized in the epidermis of embryos which are maternally and zygotically mutant for *PAR-6$^{\Delta 22}$*, whereas Baz accumulates at spots the cell-cell contacts. In contrast, staining for Sdt is almost absent in the PAR-6 deficient epithelium. **C,** PAR-6 mutant follicle cells are marked by the absence of RFP and display loss of PATJ at the apical cell-cell contacts. **D**, Sdt and PATJ fail to coimmunoprecipitate with PAR-6 under endogenous levels in embryonic lysates. **E**, Vice versa, Sdt and aPKC but not PAR-6 co-immunoprecipitate with GFP-Crb. **F,** Downregulation of PAR-6 in S2R+ cells results in decreased aPKC expression and an abolished aPKC activity (pT555 = autophosphorylation site) and Baz phosphorylation (Baz pS980). **G,** Expression of a constitutively active aPKC-variant does not rescue loss of Sdt in *PAR-6$^{\Delta 22}$* mutant follicle cells. **H,** In aPKC-mutant embryos PATJ colocalizes with Baz at (ectopic) cell-cell contacts. Scale bars = 5µm in A, B, H; 10µm in C and G.

**Figure 2 PAR-6 prevents proteasomal degradation of Sdt**. **A**, Western Blot analysis of maternally and zygotically mutant *PAR-6$^{\Delta 22}$* embryos in comparison to wild-type embryos. **B,** Inhibition of the proteasome by expression of RNAi against Rpn2 stabilizes Sdt expression in *PAR-6$^{\Delta 22}$* mutant follicle cells, whereas expression of smurf-RNAi does not (**C**). Scale bars = 10µm.

**Figure 3 PAR-6 regulates Sdt stability via Rpn13**. **A,** Downregulation of Rpn13 restores Sdt localization at apical junctions in *PAR-6$^{\Delta 22}$* mutant follicle cells. Scale bar = 10µm.

**Author contributions**

M.P. Krahn and A. Sen designed and performed the experiments and wrote the manuscript.

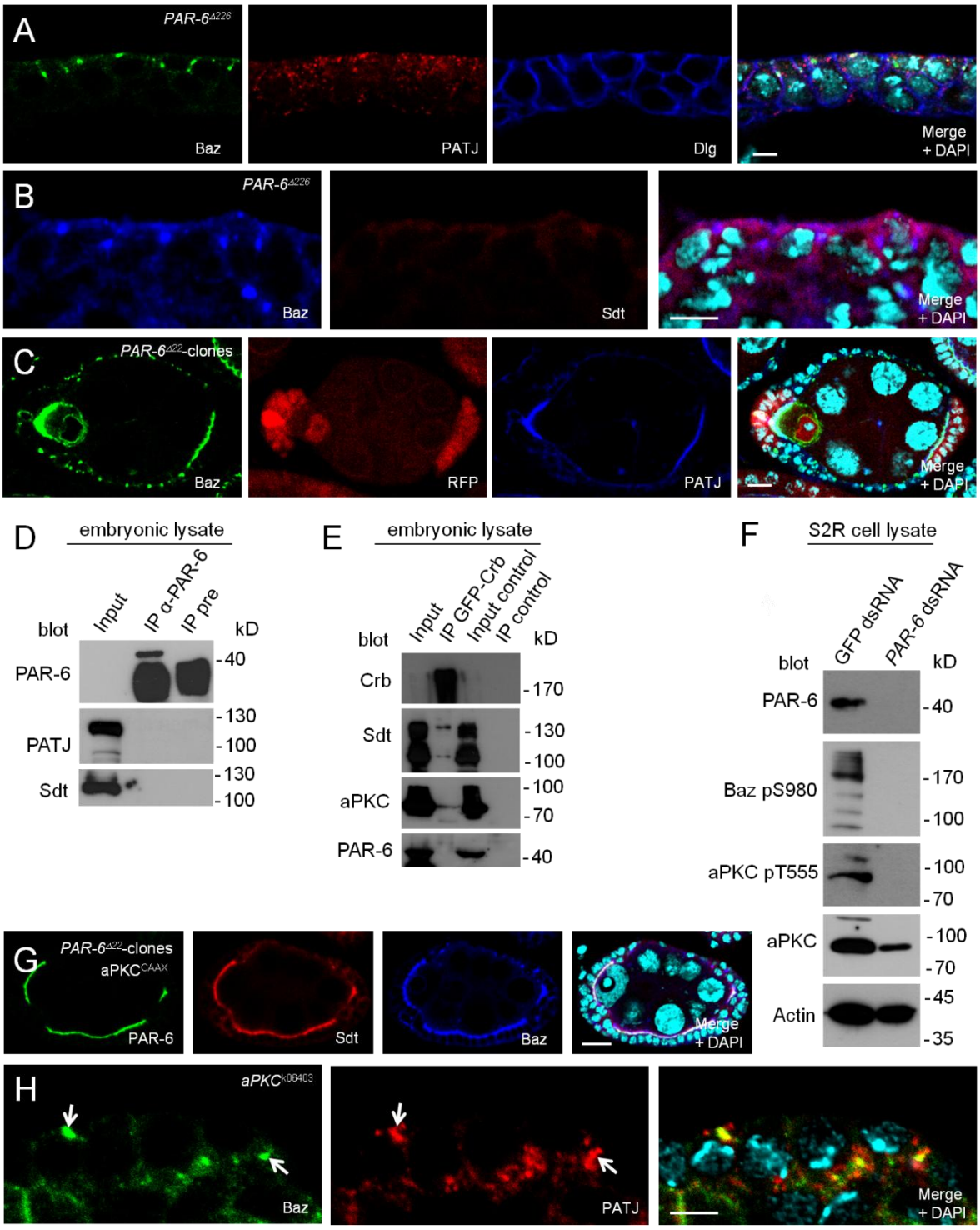
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**Figure 1**

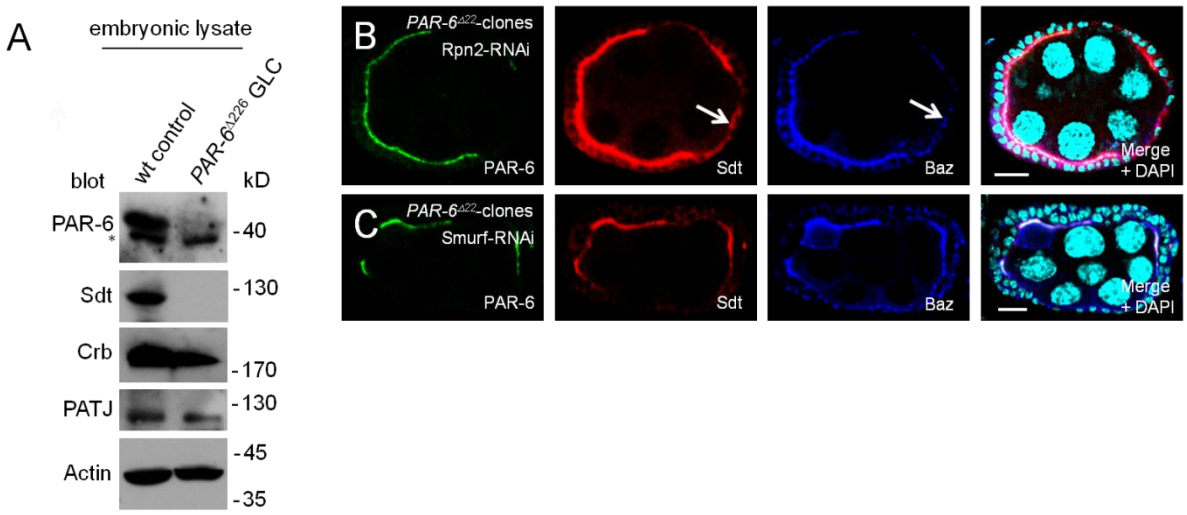


**Figure 2**

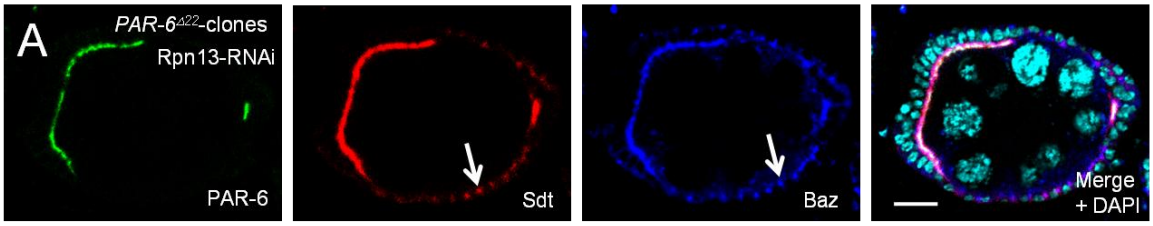


**Figure 3**

# 4. Discussion

## 4.1 Upstream regulation mechanisms of PATJ

In the past years extensive research in the field of cell polarity has unveiled many key genes which have been shown to play important roles in the establishment and maintenance of cell polarity. This study has been concerned mainly with apical-basal polarity where different proteins or protein complexes seem to work in tandem in developing polarity in a polarized tissue. On the apical side of the cell two important complexes namely PAR/aPKC complex and Crumbs acts in a hierarchical manner to establish apical polarity with proper formation of cell-cell contacts. This process is antagonized by a series of other proteins: Dlg, Scrb and Lgl which localize to the basal-lateral membrane. A dynamic interplay between these apical and basal complexes results in the development of adjacent polarized cells with cell-cell contacts. Although it is still not clear how these mechanism works to mutually exclude each other in localizing and stabilizing the polarity, research from the past have shown that it depends on cell types and also on the developmental stages.

In early *Drosophila* epithelia the Crumbs complex which localizes at the SAR just above the ZA helps to establish cell polarity by binding to the N-terminal region of Sdt via its PDZ domain. The third component of this complex PATJ has been supported by many evidences in previous research irrespective of tissue types (Klebes and Knust, 2000; Roh et al., 2002b). In photoreceptor cells of Drosophila eyes PATJ seems to be indispensable in stabilizing the Crumbs complex (Richard et al., 2006). Our studies have shown different results in elucidating the role of PATJ in other cell types, the epithelial ectoderm and the follicular cell epithelia.

In mature epithelia PATJ have been shown to preliminarily express on the tip of the invaginating membrane during early embryogenesis. As the cell starts to mature with the onset of gastrulation, PATJ is seen to localize at the cell-cell junction along with the proposed

other two members of the Crb complex, Crb and Sdt. Though it was not clear what mechanisms could have been involved in different localization patterns of PATJ and also what factors are responsible for recruiting PATJ to the cell junctions in the later stages, our research has shed some light on this issue. In our immune-localization experiments on fly embryos lacking different proteins (maternal and zygotic components) we found that in contrast to previous assumptions, Crb is not essential for recruiting PATJ to the apical junctions as PATJ remains correctly localized in the absence of *crumbs* during gastrulation until the later stages when cell polarity is severely disturbed and Baz becomes mislocalized. Interestingly in maternal or zygotic *bazooka* mutants, PATJ is normally localized during cellularization but with maturation of the epithelium PATJ is mislocalized from the apical cortex bringing up the question if PATJ is targeted correctly by Baz. Co-immunoprecipitation assay from wild type embryonic lysates have shown that endogenous PATJ is precipitated along with Baz and Sdt. Recent studies have shown that Sdt binds directly to Baz (Krahn et al., 2010a). Since PATJ have been reported to bind Sdt via its L27 domain, we investigated whether Baz-Sdt complex targets PATJ to the apical membrane. PATJ localization in *sdt* mutant embryos have confirmed our hypothesis. PATJ looses its proper localization at the apical junction in a *sdt* mutant background on later embryonic stages while PATJ localization during cellulaurization is not disturbed mostly because Sdt is expressed in the later stages of embyronic development. Taken together Sdt have been shown to correctly target PATJ to the apical junction of epithelial cells. This observation have been supported by our chimeric construct where the indespensable L27 domain of PATJ have been replaced by the PDZ binding domain of Sdt, $PATJ_{\Delta L27\text{-}PDZ(Sdt)}$ expressed under the ubiquitin promoter, thereby establishing a direct interaction to Crb. Although major fraction of the chimeric protein is cytosolic it can rescue PATJ null mutant to a certain extent. To further elucidate if PATJ directly interacts to Baz/Crb via PAR6 we tested yet another chimeric construct where the L27 domain of PATJ is replaced by the PDZ binding domain of PAR-6, $PATJ_{\Delta L27\text{-}PDZ(PAR6).}$ PAR-6 is known to bind to both Crb (Lemmers et al., 2004; Kempkens et al., 2006) and Baz

(Joberty et al., 2000; Lin et al., 2000) and that has been reflected in our result as the expression of the chimeric protein leads to its more junctional localization and also rescues the null mutant. These observations have shown that PATJ associates with both Crb-Sdt and Baz-Sdt complexes at different time points of development for its functionality and the capability mostly depends on its L27 domain.

In *Drosophila* secondary epithelia, the follicle cell epithelium of the *Drosophila* ovary we found a different situation: in *crb* mutant follicle cells PATJ and Sdt are totally absent from the apical junctions. However this might be due to the fact that Baz is mislocalized in *crb*-mutant follicle cells, too. In comparison loss of Sdt or Baz from follicle cells gives us the same result like the embryonic epithelium with complete mislocalization of PATJ. Unlike embryonic epithelium, follicular epithelial cells show intermediate phenotype (where PATJ localization only depends on Baz-Sdt complex and not Crb-Sdt) and photoreceptor cells in *Drosophila* eye (where PATJ localization is dependent on Crb (Richard et al., 2006)). Vice versa PATJ is found to stabilize Crb-Sdt complex in photoreceptor cells (Nam and Choi, 2006; Richard et al., 2006; Zhou and Hong, 2012). These data suggests that function and localization of PATJ varies from cell types studied.

One interesting question that needs still to be elucidated is how PATJ is localized to the furrow canal during early embryogenesis. Probable factors which are known to be expressed in the furrow canal are Slam and Nullo which functions through recycling endosomes and centrosomes (Acharya et al. 2013) or by interaction with RhoGEF2 (Wenzl et al., 2010) and could possibly contribute to the unique localization of PATJ.

On the way to elucidate which of the conserved domains of PATJ are responsible for its functionality and proper localization to the apical cell junction, we prepared different deletion constructs tagged with C-terminal GFP. *Drosophila* PATJ consists of a conserved L27 domain at its N-terminal (which binds to Sdt) followed by 4 PDZ protein-protein interaction domains. To avoid artificial overexpression of the deletion constructs, they have been

expressed under the ubiquitin promoter, which results in a close-to-endogenous protein expression. The chimeric proteins are expressed in a *PATJ*-mutant background to check for the transgenic proteins' localization and if they can rescue the *PATJ* null mutant phenotype. Since from previous studies in mammalian cells, PATJ have been found to bind Pals1 at the TJ via a heterodimerization of their L27 domains (Roh et al., 2002; Li et al., 2004; Straight et al., 2004), it would have been interesting if the L27 domain of *Drosophila* PATJ also display the same functionality. Likewise deletion of the L27 domain of PATJ ($PATJ_{\Delta L27}$-GFP) prevents the cortical localization of the protein as it fails to bind to Sdt. Consistently $PATJ_{\Delta L27}$-GFP cannot rescue the null mutant rendering the importance of the L27 domain for the protein's functionality. Deletion of any one of the PDZ domains ($PATJ_{\Delta PDZ1}$-GFP, $PATJ_{\Delta PDZ2}$-GFP, $PATJ_{\Delta PDZ3}$-GFP and $PATJ_{\Delta PDZ4}$-GFP) however doesn't affect the subcellular localization of the modified proteins. Even these truncated proteins are more or less fully functional on a mutant background. This suggests that none of the PDZ domains is in fact necessary for the proper localization or functionality. However, evaluation of the hatching rates has revealed that PDZ1 is of more importance than the other PDZ domains. According to earlier reports that a truncated version of PATJ can replace the protein's functionality (Nam and Choi, 2006; Richard et al., 2006; Penalva and Mirouse, 2012), we aimed to map down the exact location of the protein responsible for its functionality. As mentioned earlier deletion of the L27 domain resulted in complete loss of localization and functionality, while deletion of all the four PDZ domains leaving the L27 domain intact shows mostly cortical localization of the protein but looses the rescue capacity. Having a look further in combining the L27 domain with one of the PDZ domains was the next aim. Interestingly in rescue experiments we found that the L27 domain coupled to the first PDZ domain ($PATJ_{L27\text{-}PDZ1}$-GFP), most of the flies die in the late pupal stages with complete morphogenesis hinting to the fact that it rescues the pupal lethality but fail to hatch. Upon overexpression of $PATJ_{L27\text{-}PDZ1}$-GFP flies were able to hatch but are sterile, indicating that the female germline development is affected. In contrast to the above result presence of the second PDZ domain along with the L27 and the first PDZ

domain $PATJ_{L27\text{-}PDZ1\text{-}PDZ2}$-GFP can fully rescue the PATJ null mutant phenotype and survived flies are fertile. Upon deletion of the first PDZ domain ($PATJ_{L27\text{-}\Delta PDZ1\text{-}PDZ2}$-GFP) this rescue capacity has been lost once again. Taken together it depicts that multiple PDZ domains of PATJ along with the L27 domain might contribute in redundancy towards the functionality of the protein.

### 4.2 The role of PAR-6 in PATJ localization and stabilization of the Crb complex

Since PATJ have been now shown to be a part of both Baz-Sdt (presumably early embryonic stages) and Crb-Sdt (presumably late embryonic stages), we tried to investigate possible mechanisms which might trigger the release of Sdt from Baz-Sdt complex making Sdt available for the Crb-Sdt complex instead. It was shown by Krahn et al. that upon phosphorylation by aPKC on Baz at Serine 980, Baz releases Sdt to bind to Crb (Krahn et al., 2010b). In turn PAR-6 binds to aPKC, modulating its kinase activity (Suzuki and Ohno 2006). Strikingly we found that PATJ, Sdt and Crb are mislocalized in PAR-6 mutant embryos and follicle cells. Previous studies have reported an interplay between the Crb and PAR/aPKC complexes (Hurd et al., 2003; Kempkens et al., 2006; Penkert et al., 2004; Krahn et al., 2010; Wang et al., 2004). In line with their findings we hypothesized that PAR-6 might associate with and thereby stabilizes the Baz-Sdt-PATJ and the Crb-Sdt-PATJ complexes. Co-immunoprecipitation from wild type embryonic lysates did not confirm this hypothesis as endogenous PATJ or Sdt could not be detected upon immunoprecipitation of PAR-6. Another possible explanation would be that PAR-6 modulates the phosphorylation of Baz which in turns influences the Baz-Sdt assembly. Thus in the absence of PAR-6, Baz phosphorylation by aPKC should be enhanced. However immunoblotting on deficient S2R+ cells as well as PAR-6 mutant embryonic lysate with an antibody directed towards phosphroylated BazS980 failed to show any increase in the phosphorylation in comparison to wild type, disqualifying this hypothesis. A third possibility was suggested by the observation that is Sdt not only

mislocalized but its expression is dramatically reduced or even absent in PAR-6 mutant embryos. Degradation of Sdt in turn would explain why PATJ and Crb are mislocalized in PAR-6 mutant cells. One possibility of Sdt loss could be via proteosomal degradation. Indeed, a proteasomal receptor (Rpn13) was found in a screen to interact directly with the PDZ-domain of PAR-6 in *C. elegans* (Lenfant et al., 2010). So current experiments are ongoing to prove if PAR-6 interacts with Rpn13 in *Drosophila* and helps to prevent of degradation of Sdt. We already found that inhibition of either core components of the proteasome (Rpn2) or Rpn13 in *PAR6* mutant follicle cells results in a stabilization of Sdt at the apical junctions.

In mammalian cells Rpn13 is reported to recruit the deubiquitinaliting enzyme UCH37 (Ubiquitin carboxy-terminal hydrolase 37) which associates with the 26S proteasome (Hamazaki et al., 2006). In future studies we want to further investigate if inhibition of UCH37 leads to the rescue of the degradation of Sdt. Since loss of Rpn13 has been reported to have no strong phenotype we want to test whether loss of PAR-6 and Rpn13 in early embryonic stages can rescue Sdt.

### 4.3 PATJ and its role in cell polarity and beyond

Since the discovery of the Crumbs complex in Drosophila where Crb has been reported to bind to Sdt (Bachmann et al., 2001; Hong et al., 2001), extensive research have been made to find out the functions of the proteins of the Crb complex. Although Crb and Sdt have been studied to have role in establishing and regulating apical-basal polarity, much less have been known about the third component of the complex, PATJ. Previous studies described the role of PATJ in an ambiguous manner. In mammalian cell culture, knockdown of PATJ results in loss of Pals1 and reduction in the TJ assembly (Michel et al., 2005; Shin et al., 2005). mPATJ also plays a role in cell migration (Shin et al., 2007). In *Drosophila* PATJ has been controversially reported to play in regulation of cell polarity (Djiane et al., 2005; Nam and Choi, 2006; Pielage et al., 2003; Richard et al., 2006; Tanentzapf et al., 2000). To verify the

facts that PATJ do play a role in cell polarity we generated *PATJ* null mutant flies via homologous recombination. Embryonic epithelia deficient of zygotic and maternal PATJ show normal localization of other cell polarity and adherent junction markers pointing to the fact that PATJ does not play a predominant role in establishing apical-basal cell polarity. Even looking at the secondary epithelium (follicular epithelium) *PATJ* deficient cells do not show a mislocalization of the other members of the Crb complex, but rather a reduction in the protein levels localized to the apical junction. However most of the *PATJ* mutant flies die at the early pupal stages due to morphogenetic defects during development. Further investigation on this phenotype demonstrates that a certain percentage of *PATJ* mutant embryos show defects in germ band retraction and head development.

Many cell polarity proteins have been known to regulate and stabilize cell junctions. These proteins are mainly localized to the tight junctions in mammalian cells or to the SAR in *Drosophila*. On the other hand stabilization of the AJ by Actin-Myosin cytoskeleton is another prerequisite for cell polarity. In our study *PATJ*-null mutant embryos show defects in germband retraction, one of many cell locomotions controlled by the Actin-Myosin cytoskeleton. Simultaneously Myosin-II (immunostained for Zip and Sqh) has been found to localize near the AJ, overlapping with PATJ. These observations lead us to test whether PATJ plays any role in the development or maintenance of AJ. Although morphogenetic defects arise from the deletion of PATJ, immunostainings have shown that Zip and Sqh are still properly localized in the mutant embryos. Interestingly on weakening of the AJ by removing one copy of the *shotgun* gene, encoding E-Cadherin in *Drosophila* first leads to loss of Myosin-II and eventual breakdown of the AJ as the embryo matures. Epithelial morphology is also severely disrupted as multi-layered epithelium is formed with Baz and other apical markers drifted to the cytoplasm, disrupting apical-basal polarity. This suggests that PATJ plays a strong role in Myosin dependent AJ stability.

To investigate further how PATJ influences the Myosin dependent AJ assembly, we postulate three hypotheses: 1. PATJ directly binds to Myosin-II; 2. PATJ indirectly binds to Myosin-II and 3. PATJ modulates Myosin-II activity. Previous mass spectrometry hits for PATJ interaction partners reveal MBS as a potential target. Since MBS is known to regulate Myosin-II dynamics we tested whether PATJ binds to MBS and subsequently modulates Myosin-II dynamics. Biochemical and genetic assays revealed that PATJ directly binds to MBS and therefore modulate the dynamics by inhibiting the dephosphorylation of Myosin-II by PP1cδ, resulting in an increased phosphorylation of Myosin-II. These findings were supported by a partial rescue of the pupal lethality of the *PATJ*-null mutant when deletion of one copy of the *mbs* gene was introduced. In order to find out if increased phosphorylation of Myosin-II is the sole reason for accumulation of Sqh in cell junctions, PATJ is overexpressed in embryos expressing the non-phosphorylatable Myosin-II. Surprisingly non-phosphorylatable Sqh is still stabilized upon PATJ overexpression bringing up the question if PATJ also directly recruits Myosin-II to the apical junctions. Biochemical evidences have proven a direct interaction of PATJ with Sqh supporting this hypothesis.

Although we have found a mechanism for PATJ regulating Myosin-II dynamics, there might be other redundant processes in different cell types. One of them could be the interaction of PATJ with the PDZ domain of PAR-6 (Nam and Choi, 2003) and thereby linking to Cdc42 resulting in a modulation of the Actin-myosin cytoskeleton and AJ through Cdc42 activity (Samarin and Nusrat, 2009).

Since in our PATJ mutant we have not detected any other morphological defects in other events like cellularization, germ band extension or dorsal closure we believe that PATJ plays a rather subtle role in most Myosin-II driven processes. Further investigation will be required to find out if PATJ plays no role at all in these processes or whether it is masked by other factors which might act redundantly together with PATJ. It is likely that other AJ associated proteins can act in redundancy with PATJ, namely Vinculin, an Actin binding protein or

p120-catenin. Both of these genes have been reported to stabilize AJ assembly in mammalian cells (Xu et al., 1998; Zemljic-Harpf et al., 2007). Both Vinculin and p120-catenin have been reported to be non-essential in *Drosophila* (Alatortsev et al., 1997; Myster et al., 2003). Other well known AJ-associated proteins like alpha-catenin and beta-catenin (armadillo in *Drosophila*) cannot be also ruled out of the possibility of having a redundant role with PATJ in recruiting junctional Myosin-II. Finally PATJ seems to play an essential role during metamorphosis, a process which is again based on intensive morphogenetic movements and Actin-Myosin-driven mechanisms. Consequently, further experiments need to be done to elucidate how exactly PATJ is implicated in this process.

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# 6. Appendix

## 6.1 Abbreviations

| | |
|---|---|
| aPKC | atypical protein kinase |
| ADP | Adenosine Diphosphate |
| ATP | Adenosine Triphosphate |
| Arm | Armadilo |
| AJ | Adherens Junction |
| Baz | Bazooka |
| Crb | Crumbs |
| Cont | Contactin |
| Cora | Coracle |
| Dlg | Discs large |
| Dlt | Discs lost |
| *Drosophila* | *Drosophila melanogaster* |
| GFP | Green fluorescent protein |
| Gli | Gliotactin |
| JAM | Junctional adhesion molecules |
| Kune | Kune-kune |
| Lac | Lachesin |
| Lgl | Lethal (2) giant larvae |
| MBS | Myosin binding subunit |
| MDCK | Madine-Darby canine kidney |
| Mega | Megatrachea |

| | |
|---|---|
| MHC | Myosin heavy chain |
| MLC | Myosin light chain |
| MRLC | Myosin regulatory light chain |
| MRLCK | Myosin regulatory light chain kinase |
| Nrg | Neuroglian |
| Nrx-IV | Neurexin-IV |
| PATJ | Pals-1 associated tight junction protein |
| PAR | Partition defective |
| PCP | Planar cell polarity |
| PDZ | **P**sd95, **D**isc large, **Z**O-1 |
| PP1cδ | Protein phosphatase 1cδ |
| RhoGEF | Rho dependent guanine nucleotide exchange factor |
| ROCK | Rho associated protein kinase |
| SAR | Sub apical region |
| Sdt | Stardust |
| Sinu | Sinuous |
| SJ | Septate junction |
| Sqh | Spaghetti squash |
| TJ | Tight junction |
| Vari | Varicose |