

# *Drosophila* PATJ supports adherens junction stability by modulating Myosin light chain activity

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The assembly and consolidation of the adherens junctions (AJs) are key events in the establishment of an intact epithelium. However, AJs are further modified to obtain flexibility for cell migration and morphogenetic movements. Intact AJs 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 (PSD95, Discs large, ZO-1) domain-containing protein PATJ (Pals1-associated tight junction protein) was not per se crucial for the maintenance of

apical–basal polarity in *Drosophila melanogaster* 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 worms 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 beltlike 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 (TJs; Shin et al., 2006; Chiba et al., 2008). 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 melanogaster*), 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 PAR-6 and the atypical PKC (aPKC) to the junction (Suzuki and Ohno, 2006). Although invertebrates such as *Drosophila* do not express Occludins and therefore do not develop TJs, the components of the Crb complex are localized to the TJ analogues region (often addressed as the subapical region; Tepass, 1996; Bachmann et al., 2001; Harris and Peifer, 2005), 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, 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 nonmuscle Myosin II (henceforth Myosin) cross-links Actin filaments and consists of a homodimer of two Myosin heavy chain (MHC) proteins

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Abbreviations used in this paper: AJ, adherens junction; aPKC, atypical PKC; Arm, Armadillo; Baz, Bazooka; Crb, Crumbs; DE-Cad, *Drosophila* E-cadherin; Dlg, Discs large; GEF, guanine nucleotide exchange factor; MBP, Maltose-binding protein; MBS, Myosin-binding subunit; MHC, Myosin heavy chain; Rok, Rho-associated kinase; Sdt, Stardust; *shg*, *shotgun*; Slam, Slow as molasses; Sqh, Spaghetti squash; TJ, tight junctions; Ubi, ubiquitin; Zip, Zipper.

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(encoded by *zipper* [*zip*] in *Drosophila*), which is stabilized by two Myosin essential light chain peptides (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 (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 (Mazumdar and Mazumdar, 2002), germband 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 (Vicente-Manzanares et al., 2009; Parsons et al., 2010).

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. Upon phosphorylation, Actin-induced Myosin ATPase activity is increased, and assembly competence is promoted, resulting in cross-linking of Actin filaments (Vicente-Manzanares et al., 2009). Vice versa, Myosin phosphatase, a trimeric complex of a class 1 protein phosphatase (PP1c- $\delta$ ), 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 (Shewan et al., 2005; Ivanov et al., 2007; Yamada and Nelson, 2007). In mammalian epithelial cells as well as in the *Drosophila* epidermis, Myosin accumulates at the AJ (this paper; Krendel and Bonder, 1999; Shewan et al., 2005; Ivanov et al., 2007; Yamada and Nelson, 2007); however, activation of Myosin (measured by its phosphorylation) might not occur at all AJs but predominately at newly established junctions (Yamada and Nelson, 2007). Loss of Crb and Sdt/Pals1 as well as Baz/PAR-3 and aPKC/PAR-6 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 (Müller and Wieschaus, 1996; Tepass, 1996; Suzuki et al., 2002; Mizuno et al., 2003; Harris and Peifer, 2004, 2007; Straight et al., 2004; Fogg et al., 2005; Harris and Tepass, 2008).

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 10 PDZ (PSD95, Discs large, ZO-1) 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 down-regulation 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. (2007) found that in wound-healing experiments, PATJ localizes PAR-3 and aPKC to the leading edge, suggesting a function of PATJ in cell migration.

In *Drosophila*, the role of PATJ in morphogenesis and cell polarity has been discussed controversially: An initial study describing *PATJ* as the *discs lost* gene (Bhat et al., 1999) was corrected by Pielage et al. (2003). Although PATJ was not the focus of that study, the authors found that loss of PATJ does not affect embryonic development, but because of a lack of a clean *PATJ* mutant, they did not follow up these findings. On the other hand, several studies 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 frizzles-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 pupation. 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 AJs and loss of apical–basal polarity in the epidermis. Finally, we found that PATJ directly interacts with the MBS of Myosin phosphatase and coregulates 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 (Fig. 1, A–E; Bhat et al., 1999), 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 not depicted). 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 not depicted), 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 not depicted).

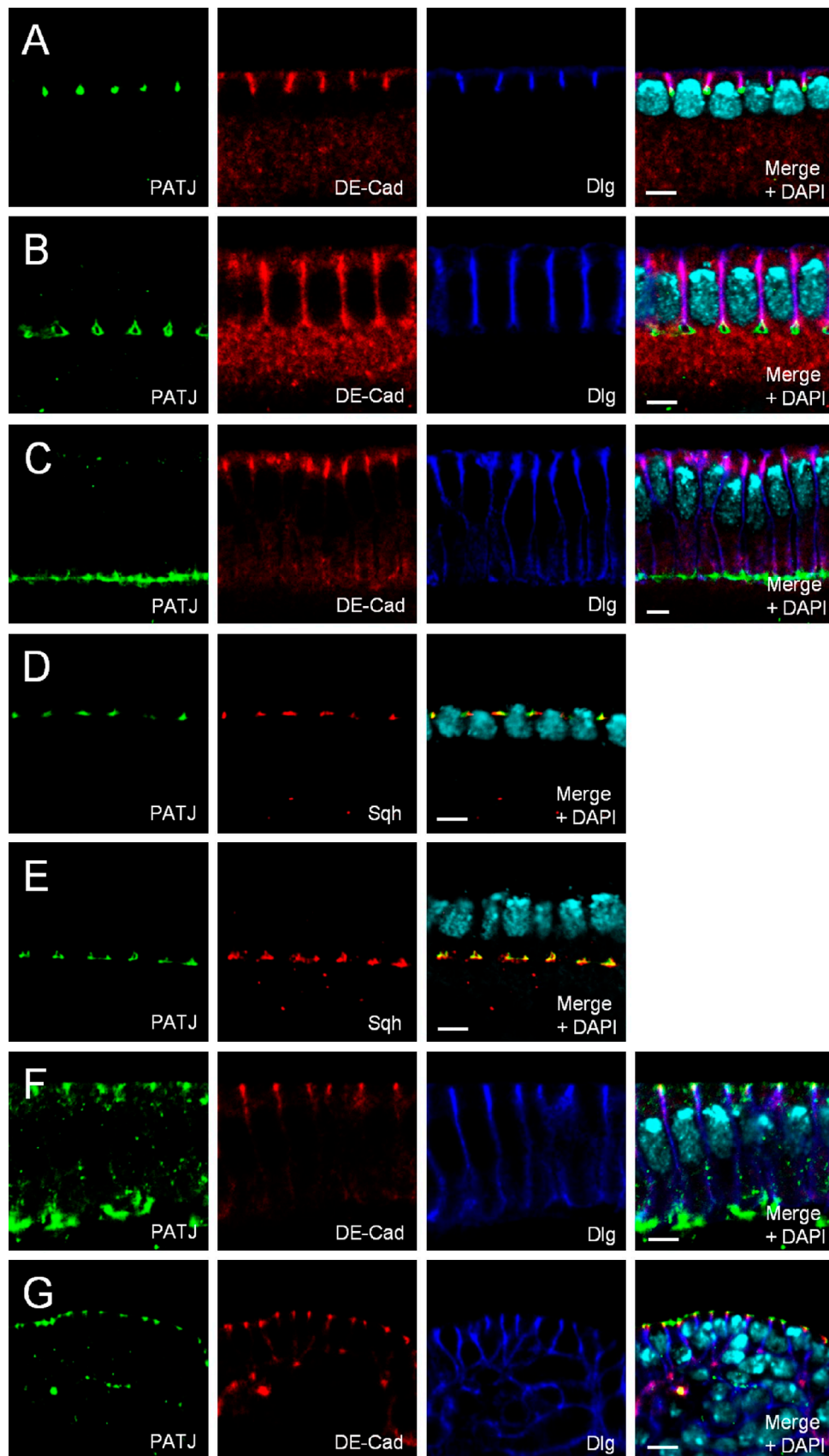
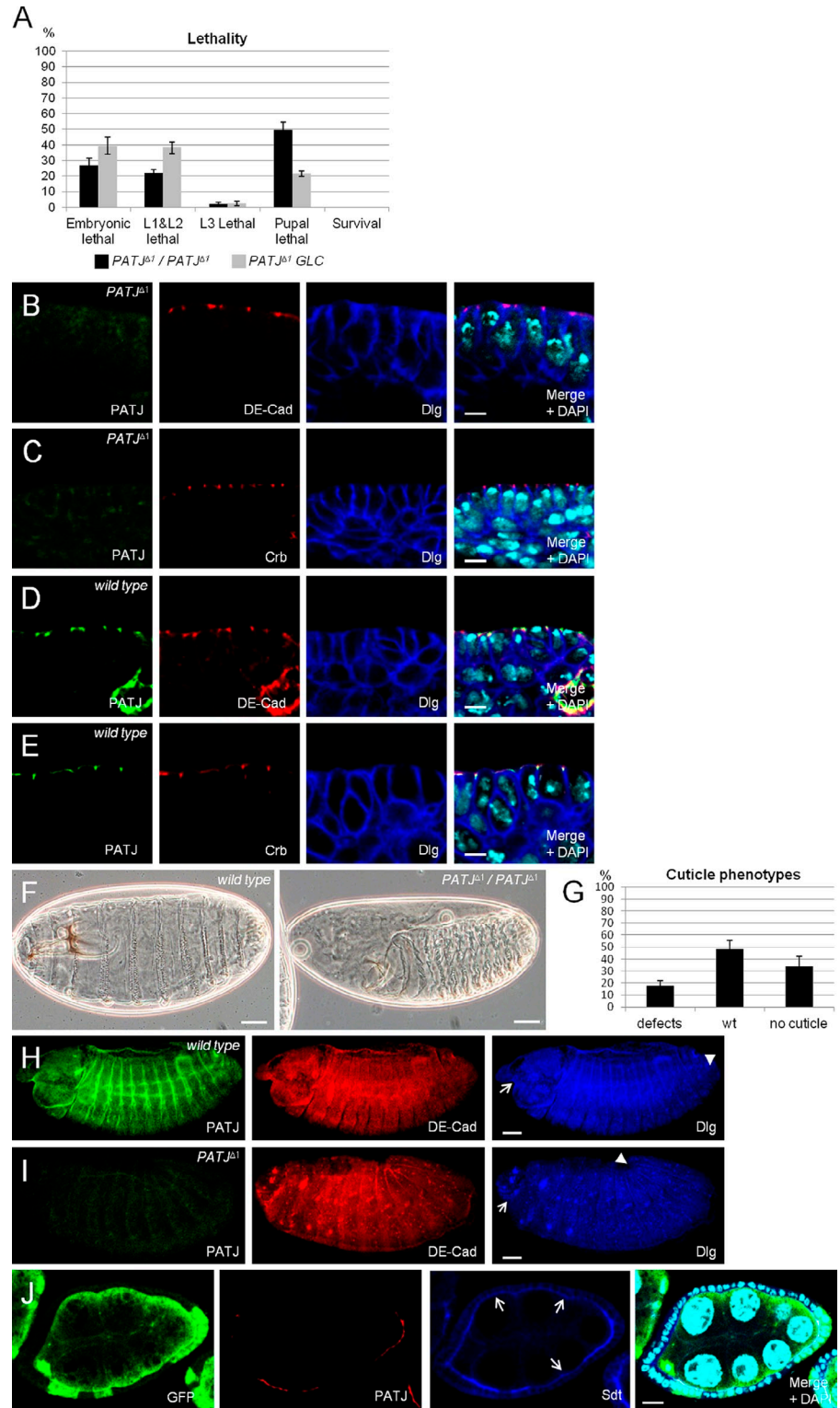


Figure 1. **Localization of PATJ during epithelial polarization.** (A–E) Endogenous PATJ localizes at the tip of the invaginating plasma membrane during cellularization (A–C), colocalizing with Sqh (D and E). (F and G) Upon gastrulation, PATJ is recruited to the apical AJ (F) and localizes at the apical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

**Figure 2. PATJ is not essential for apical-basal polarity.** (A) Lethality of flies homozygous for *PATJ<sup>Δ1</sup>*. Data were averaged from three different experiments with 100 embryos each. *PATJ<sup>Δ1</sup>/PATJ<sup>Δ1</sup>* represent embryos homozygously mutant for *PATJ* that still contain the maternal component, and *PATJ<sup>Δ1</sup> GLC* are embryos derived from *PATJ<sup>Δ1</sup>* germline clones, which lack maternal and zygotic *PATJ* expression. (B–E) Epithelia of wild-type and *PATJ<sup>Δ1</sup>* mutant embryos (derived from *PATJ<sup>Δ1</sup>* germline clones) at stages 12/13 (shown is the mature epithelium of the embryonic epidermis), stained against DE-Cad/Dlg and Crb/Dlg, respectively. (F) Cuticle phenotypes of wild-type embryos (left) and embryos homozygous mutant for *PATJ<sup>Δ1</sup>* (right). (G) Quantification of cuticle phenotypes from *PATJ<sup>Δ1</sup>* homozygous embryos. Cuticles were scored from three independent experiments with total numbers of embryos of 174. (H and I) Overview of wild-type and mutant embryos. The head region is indicated by arrows, and the posterior end of the germband is marked by arrowheads. Note that germband retraction is not completed in the embryo homozygous for *PATJ<sup>Δ1</sup>*, resulting in a posterior end at ~20% embryo length. This embryo also displays head defects. (J) Follicle cell clones for *PATJ<sup>Δ1</sup>* 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. wt, wild type. Error bars show SDs. Bars: (B–E) 5 μm; (F, H, and I) 200 μm; (J) 10 μm.



### Loss of *PATJ* results in pupal lethality

To elucidate the function of *Drosophila* *PATJ* in epithelial polarity, we established a *PATJ*-null allele (*PATJ<sup>Δ1</sup>*) 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 die 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 (unpublished data), indicating that the *PATJ<sup>Δ1</sup>* allele does not contain mutations in other genes and is a clear null allele.

Because of its strong maternal contribution, we generated *PATJ<sup>Δ1</sup>* germline 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 molasses (Slam) as markers for the invaginating plasma membrane during cellularization revealed no defects during this process in *PATJ* mutant embryos (unpublished data).

#### **PATJ does not affect apical-basal polarity**

We further analyzed apical-basal polarity in the embryonic epidermis of *PATJ<sup>Δ1</sup>* 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  $\beta$ -catenin), the apical determinants Crb, Sdt, Baz, aPKC, and PAR-6, and the lateral polarity proteins Dlg,  $\alpha$ -spectrin, and Coracle (Fig. 2, B and C compare with wild type in D and E; Fig. S2, A and B; and not depicted).

Although lethality and staining with cell polarity markers do not point to a crucial role of *PATJ* during embryogenesis, ~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 >30% of the dead embryos fail to develop any cuticle, presumably because they die before the cuticle is secreted. Immunostainings of *PATJ* mutant embryos produced similar results as cuticle preparations: ~15% of dead embryos fail to retract the germband correctly (wild-type [Video 1] and *PATJ* mutant [Video 2] embryos, expressing DE-Cad-GFP as plasma membrane marker; Fig. 2 H compare with I, head regions of the embryos are marked by arrows, and end of the germbands are marked by arrowheads). 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 caused by impaired apical-basal polarity. These embryos do not show increased apoptosis in comparison to wild-type embryos or embryos heterozygous for *PATJ<sup>Δ1</sup>* (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 AJ**

*Drosophila* embryos undergo several morphological changes during embryogenesis, including invagination of the cell membrane (cellularization), germband 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 germband 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 germband (Fig. S2 D and not depicted). Moreover, other morphological processes, such as cellularization, germband extension, and dorsal closure, are not affected in *PATJ* mutant germline clone embryos (unpublished data).

In intact epithelial cells, Myosin accumulates at the region of the AJ, colocalizing with DE-Cad (Fig. 3, A and C; and Fig. S2, E and F), but it also shows a partly overlapping localization with *PATJ*, which stains slightly more apically at the AJ (Fig. 3 A and Fig. S2, E and F). Because AJs 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<sup>R69</sup>*) 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, AJs are still intact as estimated by staining against DE-Cad and Arm (Fig. 3, B, D, and E). Later on, AJs 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, and 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 plasma membrane (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 AJs 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 MBS 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

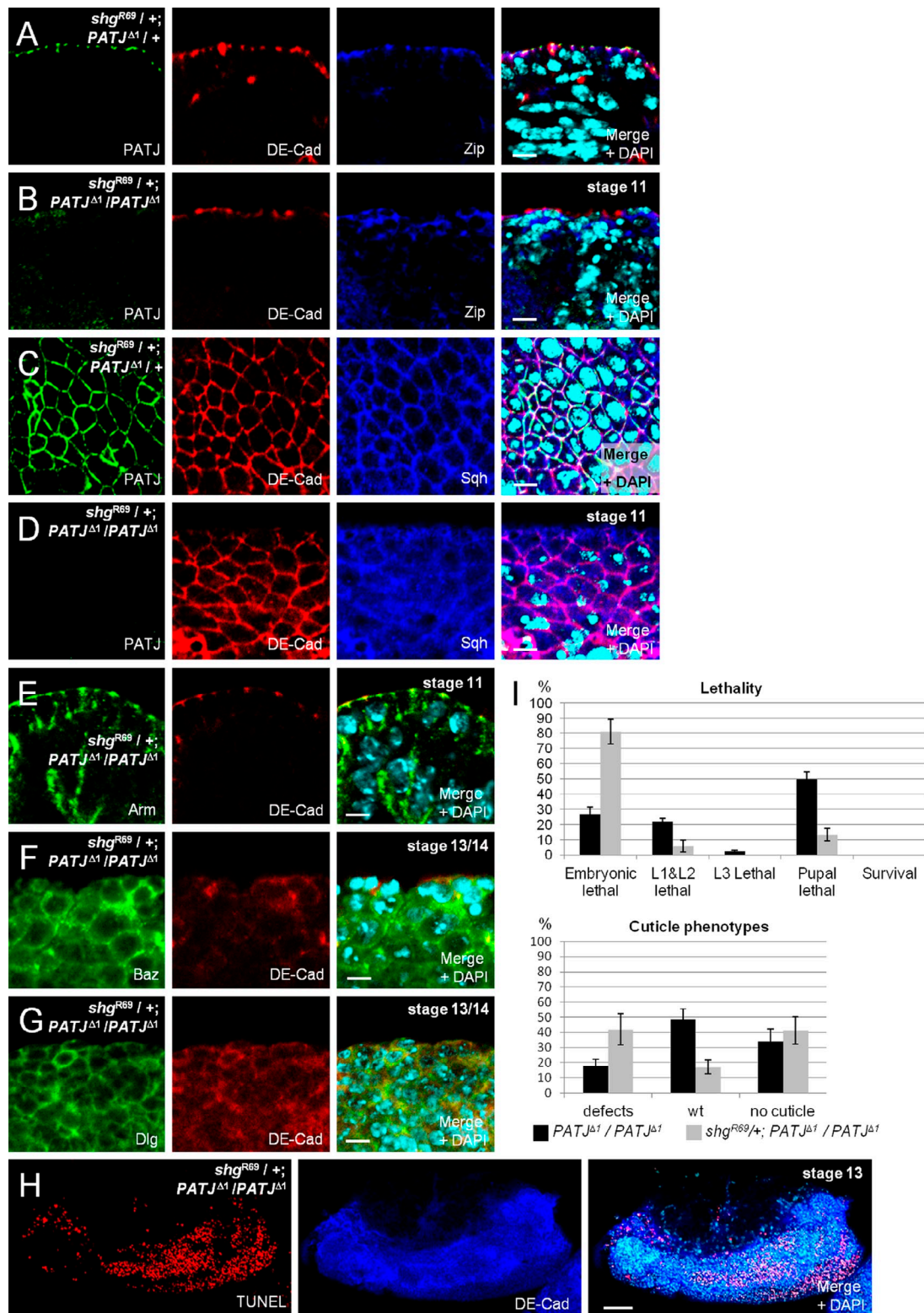


Figure 3. **PATJ supports weak AJ.** (A and C) Myosin heavy (Zip) and light (Sqh) chain accumulate at the apical junctional region in the embryonic epidermis, even in embryos expressing reduced levels of PATJ and DE-Cad (embryos heterozygous for *PATJ*<sup>Δ1</sup> and *shg*<sup>R69</sup>). (B, D, and E) Myosin is lost from AJ in embryos homozygous for *PATJ*<sup>Δ1</sup> and heterozygous for *shg*<sup>R69</sup>, although DE-Cad (B and D) as well as Arm (E) still accumulate at the *Zonula Adherens*. (F and G) In later stages of embryos homozygous for *PATJ*<sup>Δ1</sup> and heterozygous for *shg*<sup>R69</sup>, 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. Lethality data were averaged from three independent experiments with 100 embryos each. Cuticles were scored from three independent experiments with total embryos of 174 (*PATJ*<sup>Δ1</sup>/*PATJ*<sup>Δ1</sup>) and 272 (*shg*<sup>R69</sup>/*shg*<sup>R69</sup>; *PATJ*<sup>Δ1</sup>/*PATJ*<sup>Δ1</sup>). wt, wild type. Error bars show SDs. Bars: (A–G) 5 μm; (H) 200.

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 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 (Mizuno et al., 2002; Tan et al., 2003; Lee and Treisman, 2004; Mitonaka et al., 2007).

Indeed, we verified that PATJ can directly bind to MBS in vitro and associates with MBS in transfected S2R+ (Schneider 2R+) 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 (Simões 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 cytoplasm 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 not depicted).

However, we were not able to detect a significant increase in Sqh phosphorylation upon segmental PATJ overexpression (unpublished data). Only, upon the introduction of one mutant allele for *mbs*, phosphorylated Sqh is up-regulated in stripes with PATJ overexpression (Fig. 4 G and Fig. S3 C, control), 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 (Fig. 4 H, arrows, mutant cells are marked by the absence of GFP).

#### Reduced MBS activity partly rescues the *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<sup>Δ1</sup>* from 28 to 12% (Fig. 5 A). Furthermore, cuticle phenotypes (head defects and shortened cuticle) of *PATJ<sup>Δ1</sup>* mutant embryos are strongly decreased in a background heterozygous for a mutant *mbs* allele (Fig. 5 B). Interestingly, pupae homozygous mutant for *PATJ<sup>Δ1</sup>* and heterozygous for

*mbs<sup>T541</sup>* 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<sup>Δ1</sup>* homozygous mutants, a similar percentage of flies survive until pupation. However, disc shape and morphology are not as elaborated as in pupae heterozygous mutant for *PATJ* (Fig. 5 C) or in wild type (not depicted). In contrast, wing discs in *PATJ<sup>Δ1</sup>* homozygous mutant animals appear normal in L3 larvae and in very early pupae but do not undergo morphological changes and finally disintegrate shortly after pupation (Fig. 5 C). In older *PATJ<sup>Δ1</sup>/PATJ<sup>Δ1</sup>*, *mbs<sup>T541</sup>* 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 cytoplasm, 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

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 nonphosphorylatable version of Sqh (ubiquitin [Ubi]::SqhAA-GFP). Surprisingly, SqhAA-GFP is similarly recruited to/stabilized at the apical junctions as its wild-type counterpart (Fig. 6 A and Fig. S3 A, control), 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). Because of the lack of an anti-Sqh antibody, which recognizes the endogenous protein in Western blotting, we verified that myc-tagged Sqh associates with PATJ-GFP in lysates from transfected S2R+ cells (Fig. 6 C). We further performed pull-down experiments with PATJ and Sqh expressed in and purified from *Escherichia coli* and found GST-PATJ to bind to Maltose-binding protein (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 (unpublished data). These data indicate that PATJ forms a cadherin-independent platform for Myosin to be activated and further locally inhibits Myosin phosphatase to enhance Myosin phosphorylation and thereby activity. This hypothesis is supported by the overlapping localization of PATJ

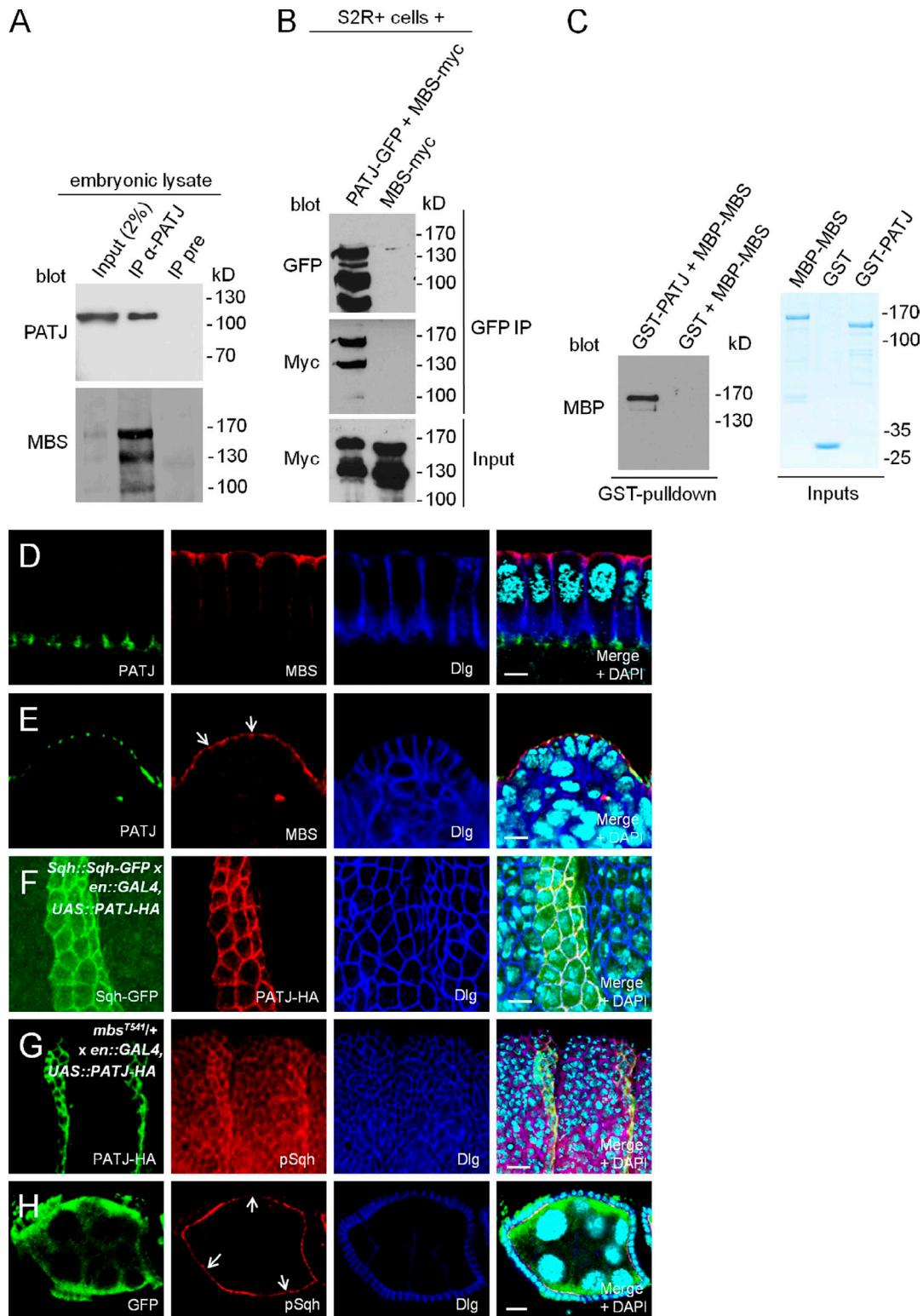


Figure 4. **PATJ enhances Myosin phosphorylation by inhibiting Myosin phosphatase.** (A) Endogenous MBS coimmunoprecipitates with endogenous PATJ from embryonic lysates. The figure represents blots from different gels with 5% (PATJ blot) and 95% of the immunoprecipitation (IP) loaded. (B) MBS-myc coimmunoprecipitates 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 and E) Localization of endogenous MBS during cellularization (D) and in mature epithelial cells of the epidermis (E; junctional MBS is marked by arrows). (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; not depicted). (G) Segmental overexpression of PATJ-HA results in an increased phosphorylation of Sqh in embryos heterozygous for *mbs<sup>T541</sup>*. (H) Follicle cell clones for *PATJ<sup>1</sup>* showing decreased phosphorylation of Sqh at the apical junction (arrows). *PATJ* mutant clones are marked by the absence of GFP. UAS, upstream activation sequence. Bars: (D–F) 5  $\mu$ m; (G and H) 10  $\mu$ m.



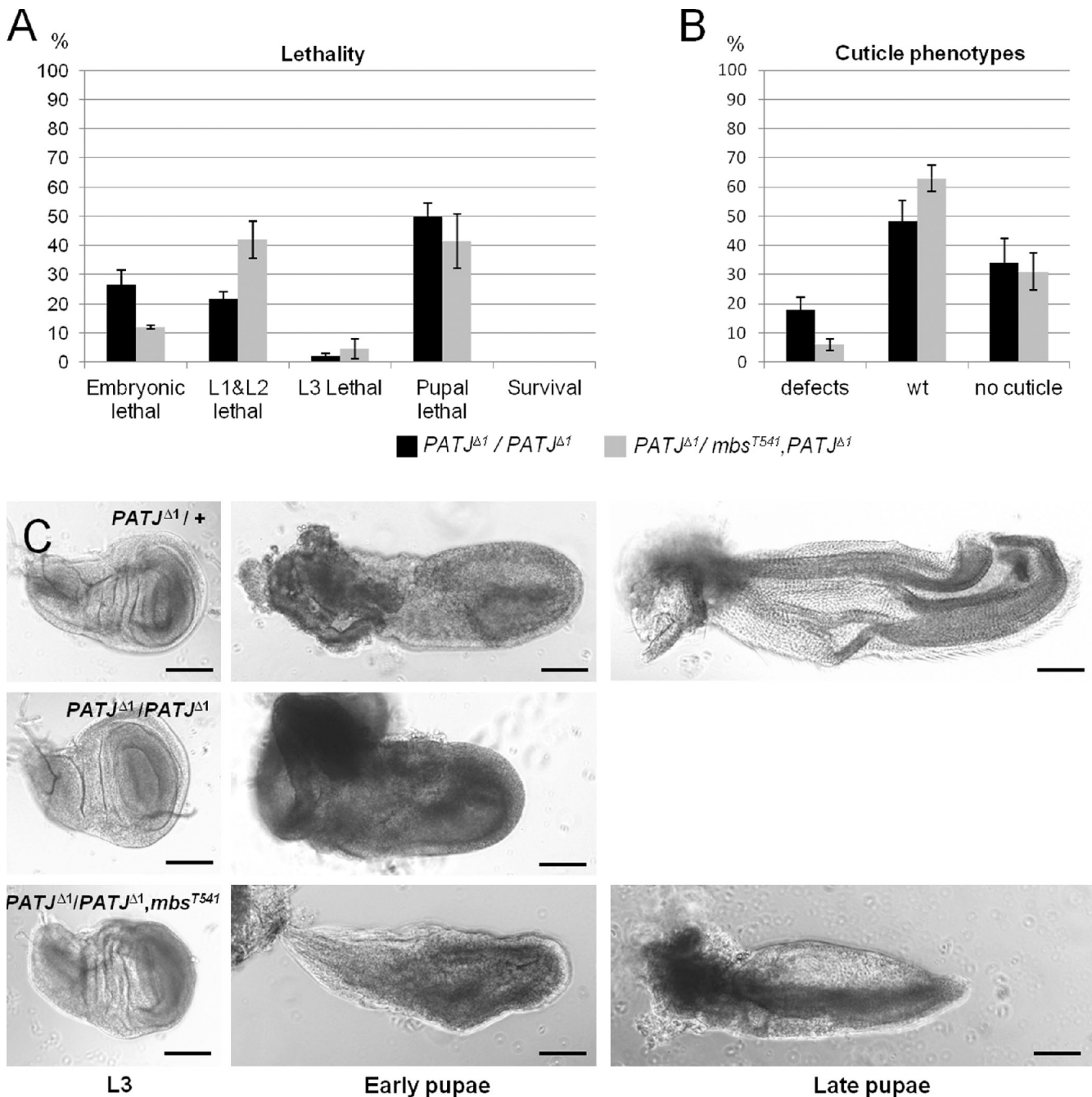


Figure 5. **PATJ and MBS interact genetically.** (A) Reduction of MBS can partly rescue the embryonic lethality of *PATJ<sup>Δ1</sup>*. Lethality data were averaged from three different experiments with 100 embryos each. (B) Embryos heterozygous for *mbs<sup>T541</sup>* and homozygous for *PATJ<sup>Δ1</sup>* show less cuticle defects than embryos homozygous for *PATJ<sup>Δ1</sup>*. Cuticles were scored from three different experiments with total numbers of embryos of 174 (*PATJ<sup>Δ1</sup> / PATJ<sup>Δ1</sup>*) and 95 (*PATJ<sup>Δ1</sup> / mbs<sup>T541</sup>, PATJ<sup>Δ1</sup>*). (C) Pupae homozygous for *PATJ<sup>Δ1</sup>* and heterozygous for *mbs<sup>T541</sup>* start metamorphosis in the imaginal discs. Note that pupae homozygous mutant for *PATJ<sup>Δ1</sup>* die soon after pupuration, resulting in autolytic tissue, in which no imaginal discs can be identified. wt, wild type. Error bars indicate SDs. Bars, 100  $\mu$ m.

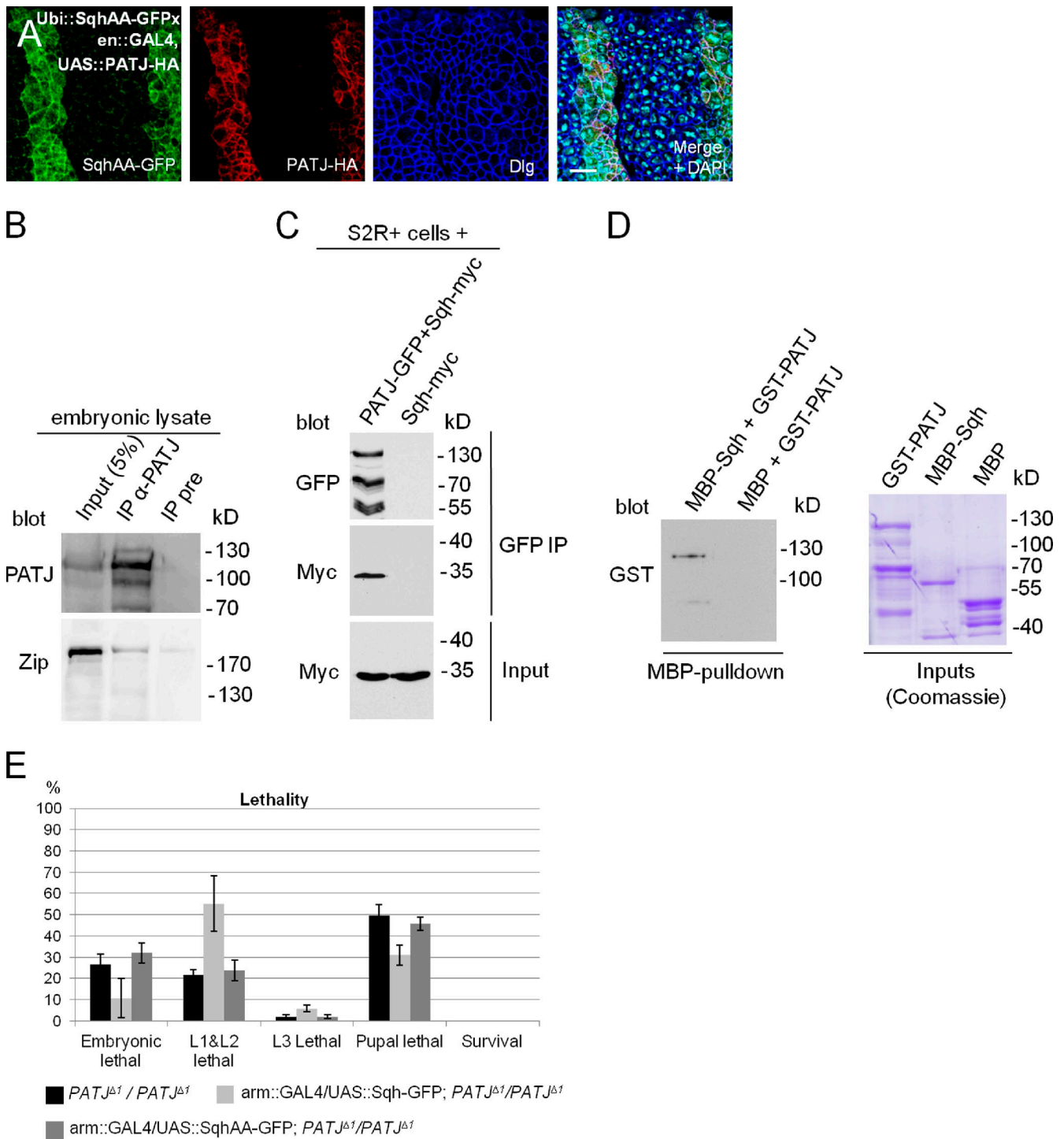
and Zip (Fig. S2 E', arrows) as well as PATJ and phosphorylated Sqh (Fig. S2 F', arrows).

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 not depicted). In contrast, overexpression of a nonphosphorylatable 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 (Shewan et al., 2005; Ivanov et al., 2007; Yamada and Nelson,



**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 (SqhAA-GFP). (B) Endogenous Zip can be copurified 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 MBP pull-down 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. IP, immunoprecipitation; UAS, upstream activation sequence. Error bars show SDs. Bar, 10  $\mu$ m.

2007). However, to accomplish cell rearrangements 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 (Roh et al., 2002; Michel et al., 2005), up to now, contradicting results obscured the role of *Drosophila* PATJ during development and in cell polarity (Tanentzapf et al., 2000;

Pielage et al., 2003; Djiane et al., 2005; Nam and Choi, 2006; Richard et al., 2006).

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 study, 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: AJs 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 germband 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 rearrangements, 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. Collectively, we suggest here a model of PATJ recruiting Myosin to the apical junctions in redundancy with other proteins, which 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 and 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<sup>Δ1</sup>* 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 caused by 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 because of a lack of a clean PATJ allele, some studies have been performed with deletions that are rescued by artificial constructs, which contained the N terminus of PATJ (Pielage et al., 2003; Nam and Choi, 2006). Other studies used RNAi-mediated down-regulation of PATJ (Michel et al. 2005; Shin et al. 2005), 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 (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 Rho guanine nucleotide exchange factor (GEF) 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 down-regulation of Lulu2 as well as of PATJ results in mislocalization of the junctional Actin–Myosin belt and impaired apical constriction. Furthermore, Shin et al. (2007) described PATJ to control cell migration in epithelial cells, which was supported by the observation that in migrating endothelial cell 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 caused by 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 (unpublished data). 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 (unpublished data; 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 caused by the fact that PATJ can directly bind the PDZ domain of PAR-6 (Nam and Choi, 2003), although the physiological relevance of this interaction needs to be further investigated in epithelial cells. Nonetheless, a physical link (via PAR-6) 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 of binding PAR-6 (and possibly indirectly Cdc42) in vitro (Hurd et al., 2003; Penkert et al., 2004; Wang et al., 2004; Kempkens et al., 2006)—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 (unpublished data).

Under physiological conditions, PATJ seems to play only a subtle or redundant role in the Myosin-dependent processes, as we did not observe any defects in cellularization, germband 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 proteins 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*: First is Vinculin, an Actin-binding protein that stabilizes AJ and focal adhesions, essential in some mammalian tissues but dispensable in the fly (Alatorsev et al., 1997; Xu et al., 1998; Zemljic-Harpe et al., 2007). Similarly, p120-catenin modulates AJ assembly in mammalian cells (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 *Zonula Adherens*, which is similar to the genetic interaction we observed between *shg*<sup>R69</sup> and *PATJ*<sup>Δ1</sup>.

## Materials and methods

### Fly stocks and genetics

The *PATJ*<sup>Δ1</sup> allele was created as previously described by Huang et al. (2008): In brief, a miniwhite gene flanked by sequences homologous to the 3.5-kbp upstream and the 3.5-kbp downstream region of the genomic region encoding the PATJ open reading frame was linearized in females using a heat shock-induced Sce-I enzyme. Homologue recombination between the linearized cassette and the PATJ genomic region took place in the female germline, resulting in progeny containing the miniwhite gene instead of the region encoding the PATJ open reading frame.

The following mutant alleles were further used: *shg*<sup>R69</sup> (strong loss-of-function allele; Godt and Tepass, 1998) and *mbs*<sup>T541</sup> (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 performed using GFP and RFP marked balancers.

*PATJ* germline clones were generated with *PATJ*<sup>Δ1</sup> recombined with FRT2A using a 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*<sup>Δ1</sup>, and homozygous mutant embryos were identified by the 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 germline transformation, (Groth et al., 2004), and attP40 and attP-VK00002 were used as landing sites. The following GAL4 lines were used: daughterless::GAL4 (#5460), engrailed::GAL4 (#6356), and Arm::GAL4 (all obtained from the 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 Resource Center) as templates using the following primers: MBS forward, 5'-CACCATGTCCTCGTGACG-3'; MBS reverse, 5'-TTTACTTAATTGCTAATACTCTAA-3'; PATJ forward, 5'-CACCATGCACCTCAGCGCGGA-3'; PATJ reverse, 5'-GTTCCGCCAGTCGGGAATCA-3'; Sqh forward, 5'-CACCATGTCATCCCGTAAGACCG-3'; and Sqh reverse, 5'-CTGCTCATCCTGTCTTG-3'.

The site-directed mutagenesis kit (QuikChange; Agilent Technologies) was used to generate defined point mutations with full-length Sqh cDNA in pENTR as a template. The following oligonucleotides were used for mutagenesis (mutation underlined): SqhAA forward, 5'-AAGCGCGCCCAACGCGCCGCGGCCAATGTGTTGCGC-3', and SqhAA reverse, 5'-GGCGAACACATTGGCCGCGCGCGTTGGGCGCGCTT-3'. Constructs were recloned into destination vectors (PWG and UWG; Murphy laboratory and *Drosophila* Genomics Resource Center) using 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).

### Immunoprecipitation and Western blotting

For immunoprecipitations, w<sup>-</sup> embryos from an overnight collection were dechorionated and lysed in lysis buffer (1% Triton X-100, 150 mM NaCl,

1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 50 mM 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 was added to the cell lysate corresponding to 500 μg of total protein. Immune complexes were harvested using protein A-conjugated agarose (BioVision), washed five times in lysis buffer, and boiled in 2× SDS sample buffer before SDS-PAGE and Western blotting. For precipitation of PATJ-GFP from S2R+ cells, GFP binder (ChromoTek) was used.

Western blotting was performed according to standard procedures. Primary antibodies used for Western blotting were as follows: guinea pig anti-PATJ (1:2,000; this study), rabbit anti-Zip (1:2,000; provided by K. Prehoda, University of Oregon, Eugene, OR; Liu et al., 2008), guinea pig anti-pSqh (1:400; provided by R. Ward, University of Kansas, Lawrence, KS; Zhang and Ward, 2011), mouse anti-α-tubulin (12G10; 1:100; Developmental Studies Hybridoma Bank), mouse antimyc (9E10; 1:100; Developmental Studies Hybridoma Bank), and rabbit anti-GFP (#A11122; 1:1,000; Life Technologies).

### 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 MBP was expressed accordingly and purified with amylose resin (New England Biolabs, Inc.). For PATJ-MBS pull-down experiments, 1 μg 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 before (see previous paragraph). For PATJ-Sqh pull-down experiments, the same protocol was applied using MBP-Sqh, GST-PATJ, and MBP alone as a negative control. Amylose resin instead of glutathione beads was used to pull-down MBP/MBP-Sqh. Rabbit anti-GST (1:10,000; Sigma-Aldrich) and rabbit anti-MBP (1:10,000; Sigma-Aldrich) were used.

### Immunohistochemistry

Embryos were fixed in 4% formaldehyde and phosphate buffer, pH 7.4, as previously described (Krahn et al., 2009). Primary antibodies used for indirect immunofluorescence were as follows: rabbit anti MBS (1:1,000; provided by Y. Nishida, Nagoya University, Nagoya, Japan; Mizuno et al., 2002), guinea pig anti-PATJ (1:500; this study), mouse anti-Sdt (1:20; provided by E. Knust, Max-Planck Institute, Dresden, Germany; Berger et al., 2007), rabbit anti-Baz (1:2,000; provided by A. Wodarz, University of Goettingen, Goettingen, Germany; Wodarz et al., 1999), rabbit anti-Zip (1:2,000; provided by K. Prehoda; Liu et al., 2008), mouse anti-Sqh (1:1,000; provided by R. Ward; Zhang and Ward, 2011), guinea pig anti-pSqh (1:100; provided by R. Ward; Zhang and Ward, 2011), mouse anti-Crb (Cq4; 1:50; Developmental Studies Hybridoma Bank), mouse anti-Dlg (4F3; 1:50; Developmental Studies Hybridoma Bank), rat anti-DE-Cad (DCAD2; 1:50; Developmental Studies Hybridoma Bank), mouse anti-GFP (3E6; 1:1,000; Life Technologies), and rat anti-HA (3F10; 1:1,000; Roche). Secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 647 (Life Technologies) were used at 1:400.

Images were taken on a meta confocal microscope (LSM 710; Carl Zeiss) using either 25× (NA 0.8; Carl Zeiss) or 63× (NA 1.2; Carl Zeiss) water objectives and ZEN 2010 software (Carl Zeiss). Images were processed using Photoshop (Adobe).

### Online supplemental material

Fig. S1 shows loss of PATJ in a 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. Videos 1 and 2 show the embryonic development of a wild-type embryo and a *PATJ* mutant embryo, respectively. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201206064/DC1>.

We thank E. Knust, Y. Nishida, K. Prehoda, U. Tepass, R. Ward, A. Wodarz, the Bloomington *Drosophila* stock center at the University of Indiana, and the Developmental Studies Hybridoma Bank at the University of Iowa for sending reagents. We thank A. Wodarz, R. Witzgall, and members of the Krahn laboratory for discussion and D. Lbk for his support in the laboratory. We are further thankful to F. Sprenger for his assistance with live imaging.

This work was supported by grants from the Deutsche Forschungsgemeinschaft to M.P. Krahn (DFG3901/1-1 and DFG3901/2-1) and by the Sonderforschungsbereich 699.

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