Drosophila Patj plays a supporting role in apical-basal polarity but is essential for viability

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SUMMARY
Patj has been characterized as one of the so-called polarity proteins that play essential and conserved roles in regulating cell polarity in many different cell types. Studies of Drosophila and mammalian cells suggest that Patj is required for the apical polarity protein complex Crumbs-Stardust (Pals1 or Mpp5 in mammalian cells) to establish apical-basal polarity. However, owing to the lack of suitable genetic mutants, the exact in vivo function of Patj in regulating apical-basal polarity and development remains to be elucidated. Here, we generated molecularly defined null mutants of Drosophila Patj (dPatj). Our data show conclusively that dPatj only plays supporting and non-essential roles in regulating apical-basal polarity, although such a supporting role may become crucial in cells such as photoreceptors that undergo complex cellular morphogenesis. In addition, our results confirm that dPatj possesses an as yet unidentified function that is essential for pupal development.

KEY WORDS: Drosophila Patj, Polarity proteins, Apical-basal polarity, Embryonic epithelium, Follicular epithelium, Photoreceptor

INTRODUCTION
Polarity proteins play evolutionarily conserved roles in regulating apical-basal polarity in epithelial cells (Wang and Margolis, 2007). Pals1-associated tight junction protein (Patj) is a multi-PDZ domain protein that binds the apical polarity protein complexes Crumbs (Crb)-Stardust (Sdt; Pals1) (Roh et al., 2002; Roh et al., 2003) and Par-6–aPKC (Nam and Choi, 2006). In Drosophila, the homolog of Patj was first identified as the product of discs lost (dlt) (Bhat et al., 1999), but Pielage et al. later showed that dlt actually encodes a protein involved in cell cycle control and the original dlt was renamed Drosophila Patj (dPatj) (Pielage et al., 2003). RNAi knockdown results suggested that dPatj is an essential gene that is required for initiating and establishing apical-basal polarity, primarily through its regulation of Crb (Bhat et al., 1999). However, Pielage et al. generated a synthetic dPatj mutant by rescuing all of the deleted genes with the exception of dPatj in a small deficiency and found it to be viable, disputing essential roles for dPatj in polarity and development (Pielage et al., 2003). Such conflicting results were partially reconciled when it was shown that this synthetic dPatj mutant is in fact a hypomorphic allele (dPatj<sup>synhypo</sup>) that expresses a truncated dPatj protein from one of the rescuing DNA fragments (Nam and Choi, 2006). They constructed a new synthetic null mutant, dPatj<sup>synnull</sup>, that was free of dPatj coding sequences and found that it was early larval lethal. Unfortunately, polarity defects could not be assessed in dPatj<sup>synnull</sup> embryos due to difficulties in removing dPatj maternal contributions (Nam and Choi, 2006).

Thus, owing to the lack of suitable genetic mutants, it remains to be determined whether Patj is indeed essential for apical-basal polarity and general development. Here, we generated multiple molecularly and genetically defined null mutants of dPatj to address this key issue.

MATERIALS AND METHODS
Drosophila stocks
y<sup>1</sup> w<sup>67c23</sup>; P{GSV2}/GS0262/TM3, Sb<sup>1</sup> Ser<sup>2</sup> (stock #204965) was obtained from the Kyto Drosophila Genetic Resource Center. Stocks obtained from the Bloomington Drosophila Stock Center: w<sup>1118</sup>; Df(3L)BSC123/TM6B, Tb<sup>1</sup> (BL#9143); w<sup>*</sup>; FRT-2A (BL#6977); w<sup>*</sup>; D<sup>1</sup> CTM3, Sb<sup>1</sup> (BL#6902); and w<sup>*</sup>; Ubi-GFP<sup>nl2</sup> Ubi-GFP<sup>nl2</sup> FRT-2A (BL#5825); w<sup>*</sup>; ovo<sup>1-18</sup> FRT-2A/s<sup>Y</sup> (BL#24867); w<sup>*</sup>; ovo<sup>1-18</sup> FRT-2A/s<sup>Y</sup> (BL#1339); ovo-FLP, w<sup>*</sup> (BL#8727); w<sup>1118</sup> ey-FLP (BL#5580); and w<sup>*</sup>; D<sup>1</sup> CTM3, Sb<sup>1</sup> (BL#6663; hereafter referred to as w<sup>*</sup>; Dr/TM3 w<sup>1118</sup> GFP). The knockout null allele crb<sup>9AVK00020</sup> (crb<sup>9AVK00020</sup>) was described previously (Huang et al., 2009).

Generation and characterization of dPatj deletion and rescue alleles
F{GSV2}/GS0262 excision mutagenesis was performed according to the standard protocol (Hummel and Klambt, 2008). dPatj<sup>33</sup>, dPatj<sup>34</sup> and dPatj<sup>7</sup> were isolated from a total of 253 excision candidates screened by Df(3L)BSC123 complementation. PCR verifications and sequencing were performed using primers (5<sup>′</sup>-H11032 CGAGACGCGGCCGCGGCAGAC-3<sup>′</sup>)/H11003 CGAGACGCTAGCCTAGTTCCGCCAG-3<sup>′</sup>). dPatj::GFP .nls<sup>3L1</sup> Ubi-GFP .nls<sup>3L2</sup> FRT-2A were integrated into attP-(BL#24867); w<sup>*</sup>; ovo<sup>1-18</sup> FRT-2A/s<sup>Y</sup> (BL#1339); ovo-FLP, w<sup>*</sup> (BL#8727); w<sup>1118</sup> ey-FLP (BL#5580); and w<sup>*</sup>; D<sup>1</sup> CTM3, Sb<sup>1</sup> (BL#6663; hereafter referred to as w<sup>*</sup>; Dr/TM3 w<sup>1118</sup> GFP). The knockout null allele crb<sup>9AVK00020</sup> was described previously (Huang et al., 2009).

Generation of somatic and germline clones of dPatj
dPatj<sup>33</sup> germline clone embryos were collected from crosses of ovo-FLP; dPatj<sup>33</sup> FRT-2A/ovo-FLP FRT-2A x dPatj<sup>33</sup> [or dPatj<sup>34</sup> or Df(3L)BSC123 or dPatj<sup>33</sup> VK20-dPatj-L/TM3, w<sup>1118</sup> GFP (Xu and Rubin, 1993; Chou and Perrimon, 1996). To generate follicular clones, young females of hs-FLP/+; dPatj<sup>33</sup> FRT-2A/ubi-GFP FRT-2A were heat shocked for 1 hour at 38°C and then aged for 2–4 days prior to ovary dissection. To generate larval imaginal disc clones, embryos from the hs-FLP; ubi-GFP FRT-2A x dPatj<sup>33</sup> FRT-2A/TM3, w<sup>1118</sup> GFP crosses were collected for 24 hours,
heat shocked for 1 hour at 38°C 1 day later, and maintained at 25°C until the third instar larvae emerged. To generate dPatj photoreceptor clones, white pupae (less than 2-3 hours into pupation) were collected from the ey-FLP; dPatj\(^{\Delta 7}\) FRT-2A/TM3 twi\(\triangleright\)GFP \(\times\) ubi-GFP FRT-2A cross and aged at 18°C until the desired pupal stages (Hong et al., 2003). Pupae were genotyped by the absence of twi\(\triangleright\)GFP.

**Immunostaining and confocal imaging**

Immunostaining of embryos, larval imaginal discs and adult ovaries (Tanentzapf et al., 2000; Hong et al., 2001; Huang et al., 2009) and pupal retina dissection and immunostaining (Hong et al., 2003) were performed as previously described. Primary antibodies were: mouse anti-dPatj (full-length) and rabbit anti-dPatj-N (the first 517 amino acids) (Huang et al., 2009) 1:500; rabbit anti-GFP (Huang et al., 2009) 1:1500; guinea pig anti-Baz (Huang et al., 2009) 1:500; rabbit and rat anti-Par-6 (Huang et al., 2009) 1:500; rabbit anti-Std (Hong et al., 2001) 1:100-200; mouse anti-Crb (Cq4; DSHB) 1:10-100; mouse anti-Arm (N2 7A1; DSHB) 1:50-100; rat anti-DE-Cad (DCAD2; DSHB) 1:100; mouse anti-Dlg (4F3; DSHB) 1:50; and rabbit anti-aPKC (Santa Cruz) 1:1000. Secondary antibodies: Cy2-, Cy3- or Cy5-conjugated goat anti-rabbit IgG, anti-rat IgG, anti-mouse IgG and anti-guinea pig IgG (Jackson Immunoresearch), all at 1:400. Images were taken on an Olympus FV1000 confocal microscope (Center for Biologic Imaging, University of Pittsburgh Medical School) and processed in Adobe Photoshop for compositions.

**RESULTS AND DISCUSSION**

**Drosophila Patj is essential for viability**

P(GSV2)G550262 is a viable P-element insertion located 303 bp upstream of the first ATG of the dPatj coding sequence (Fig. 1A). By mobilizing G550262 with transposase (Hummel and Klämbt, 2008), we recovered several imprecise excision mutants that failed to complement Df(3L)BSC123, which is a 11,312 bp deficiency encompassing the dPatj locus (Cook et al., 2012) (Fig. 1A-C and supplementary material Table S1). dPatj\(^{\Delta 7}\), dPatj\(^{\Delta 13}\) and dPatj\(^{\Delta 14}\) contained deletions that all began at the 5' UTR plus the conserved L27 motif required for the binding of Sdt or Pals1 (Roh et al., 2002; Li et al., 2004; Feng et al., 2005) (Fig. 1A-C). The 2,509 bp deletion in dPatj\(^{\Delta 7}\) further removed the first three PDZ domains that are required for interaction with Crb (Bhat et al., 1999) and Par-6 (Nam and Choi, 2003), whereas deletions in dPatj\(^{\Delta 13}\) and dPatj\(^{\Delta 14}\) also removed the first two and the first PDZ domain, respectively (Fig. 1B). Antibodies against the N-terminal and full-length dPatj proteins (Huang et al., 2009) revealed maternal contributions of dPatj in dPatj\(^{\Delta 7}\) germline clone (dPatj\(^{\Delta 7}\)/L) mutant embryos, which removed dPatj maternal contributions, dPatj staining was absent, confirming that dPatj\(^{\Delta 7}\) is a genetic null mutant (Fig. 2B-F).

Transheterozygotes among dPatj\(^{\Delta 7}\), dPatj\(^{\Delta 13}\), dPatj\(^{\Delta 14}\) and Df(3L)BSC123 suggested that the zygotic mutations of dPatj were pupal lethal, but dPatj\(^{\Delta 7}\), dPatj\(^{\Delta 13}\) and dPatj\(^{\Delta 14}\) appeared to carry additional background mutations that caused prepupal lethality (supplementary material Table S1). To confirm that the loss of dPatj specifically caused pupal lethality, we developed two independent genomic rescue lines, VK20-dPatj-L and VK20-dPatj::GFP-S, that each only contained dPatj as the single intact gene (Fig. 1B). dPatj\(^{\Delta 7}\) VK20-dPatj-L or dPatj\(^{\Delta 7}\) VK20-dPatj::GFP-S recombinants fully rescued dPatj\(^{\Delta 7}\), dPatj\(^{\Delta 13}\) and
Df(3L)BSC123 (supplementary material Table S1), dPatj\textsuperscript{VK20-dPatj-} also fully rescued the lethality of dPatj\textsuperscript{VK20-dPatj-GLC} embryos (supplementary material Table S1), indicating that the zygotic expression of dPatj is sufficient for development. It is likely that the early larval lethality observed in the dPatj\textsuperscript{synnull} mutants (Nam and Choi, 2006) was due to additional background mutations or incomplete rescue. The expression pattern of GFP-tagged dPatj from VK20-dPatj::GFP-S was indistinguishable from the dPatj immunostaining pattern (data not shown).

**dPatj is not required for establishing apical-basal polarity during embryogenesis**

During *Drosophila* embryogenesis, cellularization is the earliest event in the formation of the polarized embryonic epithelia, and one striking feature of dPatj is its dynamic association with the leading edge of the invaginating membrane in cellularizing cells (Fig. 2B) (Bhat et al., 1999). It was reported that dPatj RNAi knockdown impairs cellularization and results in shortened epithelial cells (Bhat et al., 1999), suggesting that dPatj is required in membrane invagination and the initiation of apical-basal polarity. Surprisingly, in stage 5 dPatj\textsuperscript{VK20-dPatj-GLC} embryos (n>10), we found no discernible defects in cellularizing cells in the absence of dPatj (Fig. 2B). Because dPatj\textsuperscript{VK20-dPatj-GLC} embryos completed their development through the embryonic and larval stages (supplementary material Table S1), even if the loss of dPatj caused cellularization defects that were too subtle to be observed by immunostaining, such defects were not detrimental to normal development. Our data confirm that dPatj is dispensable for cellularization and initiating apical-basal polarity.

After cellularization, establishing apical-basal polarity in polarizing embryonic epithelia requires the Crb-Sdt complex (Tepass and Knust, 1993; Bachmann et al., 2001; Hong et al., 2001). Again, RNAi knockdown of dPatj severely disrupted the apical localization of Crb and apical-basal polarization (Bhat et al., 1999). Similarly, in polarizing MDCK cells, RNAi knockdown of Patj caused the loss of Pals1 from tight junctions and delayed tight junction formation (Straight et al., 2004; Shin et al., 2005). These data support an essential role of dPatj in establishing apical-basal polarity. Nonetheless, we found that in stage 10 and 11 dPatj\textsuperscript{VK20-dPatj-GLC} embryos, both Crb and Sdt showed normal subcellular localization (Fig. 2C,D). Crb expression was slightly reduced in dPatj\textsuperscript{VK20-dPatj-GLC} embryos, but the overall pattern was clearly undisturbed (Fig. 2C). We further examined the subcellular localization of several representative polarity proteins in dPatj\textsuperscript{VK20-dPatj-GLC} embryos, including: DE-Cadherin (DE-Cad; Shotgun – FlyBase), which marks the adherens junction; Bazooka (Baz, or Par-3), which is required for
the early stage of apical-basal polarization, Par-6 and atypical PKC (aPKC), which form an evolutionarily conserved apical complex that regulates multiple polarity proteins through aPKC-mediated phosphorylation (Suzuki et al., 2001; Betschinger et al., 2003; Plant et al., 2003; Sotillos et al., 2004; Krahn et al., 2010; Morais-de-Sá et al., 2010); and Discs large (Dlg, or Dlg1), which is required for specifying the basolateral membrane domain and junctions (e.g. septate junctions). Before 40% pupal development (pd), the apical membranes of all photoreceptors are converged at the center of the ommatidium and show enriched localization of Crb, Sdt, dPatj, Par-6 and aPKC (red). Adherens junctions that form between photoreceptors show ring-like patterns that are marked by DE-Cad, Arm and Baz (green). (G-I) In dPatjΔ7 mutant photoreceptors, Arm (G) and Baz (H) show mild disruptions (examples indicated by arrows), whereas Crb and Par-6 exhibit more severe mislocalization (I, arrowheads). All samples are from 37% pd pupae. In all panels, dPatjΔ7 clones are marked by the loss of GFP (blue). Scale bars: 10 μm.

**dPatj supports Crb expression and subcellular localization in polarized epithelial cells**

The reduction of Crb expression in the absence of dPatj has also been reported previously in polarized epithelial cells, such as the follicular cells of ovaries (Tanentzapf et al., 2000). Because such studies also used a deficiency-based dPatjΔ10 allele with multiple gene deletions (Bhat et al., 1999), we generated dPatjΔ7 clones in larval imaginal discs and adult follicular cells to investigate their phenotypes in polarized epithelial cells (Fig. 3A-E). The dPatjΔ7 mutant clones in the imaginal epithelia showed mild reductions in the expression of Crb but not Baz (Fig. 3A), whereas clones in the follicular cells showed much stronger reductions in Crb expression (Fig. 3B). Therefore, dPatj appears to be specifically required to maintain high levels of Crb expression in polarized epithelial cells. Consistent with the observations of Tanentzapf et al. (Tanentzapf et al., 2000), reduced Crb expression in dPatj mutant cells did not disrupt their apical-basal polarity: other polarity markers, such as Baz, DE-Cad, Par-6 and aPKC, all appeared to be unaffected (Fig. 3A-E).

**dPatj is required for apical-basal polarity in early pupal photoreceptors**

For cells that must undergo dramatic remodeling of their apical-basal polarity, the supporting role of dPatj in maintaining the expression and subcellular localization of Crb might become crucial. In *Drosophila*, the highly polarized subcellular structures

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**Fig. 3. dPatj supports Crb expression in polarized epithelial cells and apical-basal polarity in early pupal photoreceptors.** (A) dPatjΔ7 clone (outlined) in a larval imaginal disc shows subtle reduction in Crb expression but normal expression of Baz. (B-E) dPatjΔ7 clones in follicular epithelia show more marked loss of Crb (B) but no obvious disruption of DE-Cad (C), Par-6 (D) or aPKC (E). (F) Illustration of a transverse view of an early pupal ommatidium. Before 40% pupal development (pd), the apical membranes of all photoreceptors are converged at the center of the ommatidium and show enriched localization of Crb, Sdt, dPatj, Par-6 and aPKC (red). Adherens junctions that form between photoreceptors show ring-like patterns that are marked by DE-Cad, Arm and Baz (green). (G-I) In dPatjΔ7 mutant photoreceptors, Arm (G) and Baz (H) show mild disruptions (examples indicated by arrows), whereas Crb and Par-6 exhibit more severe mislocalization (I, arrowheads). All samples are from 37% pd pupae. In all panels, dPatjΔ7 clones are marked by the loss of GFP (blue). Scale bars: 10 μm.
Genetic characterization of Patj

Fig. 4. dPatj is essential for pupal development. (A) Deformed dPatj7/Df(3L)BSC123 (dPatj7/Df) pupa at the beginning of pupation. (B, C) dPatj7/Df and wild-type pupae at 16% pd and 72% pd, respectively. (D) Pupal development in dPatj7 VK20-dPatj7/Df(3L)BSC123 is completely rescued. WT are (A–C) dPatj7 or Df(3L)BSC123/TM3 Sb twi-GFP and (D) dPatj7 VK20-dPatj7-L/TM6 Tb. In B–D, pupal cases were removed prior to imaging. Scale bars: 300 μm.

in the photoreceptor soma are generated through a complex morphogenetic process requiring many polarity proteins, including Baz, Crb, Sdt, Par-6 and aPKC (Izaddoost et al., 2002; Pellikka et al., 2002; Hong et al., 2003). It has been reported previously that in dPatjnull photoreceptors, the apparent loss of dPatj causes the disruption of apical-basal polarity in the soma (Nam and Choi, 2003; Nam and Choi, 2006). To conclusively show that such phenotypes are indeed specific to dPatj, we generated dPatj7 mutant photoreceptor clones and examined their apical-basal polarity in early pupal retina at ~37% pupal development (pd) (Fig. 3F–I). The dPatj7 photoreceptors showed no dPatj expression (Fig. 3G) but did exhibit frequent mislocalization of major apical polarity proteins. Armadillo (Arm; Drosophila β-Catenin homolog) and Baz, which marks adherens junctions, were often disrupted in the dPatj7 photoreceptors (Fig. 3G,H), in addition to the basolateral mislocalization of Par-6 and Crb (Fig. 3I). Therefore, although it is dispensable in normal epithelia, dPatj might be crucial to maintain apical-basal polarity under more complex and stringent developmental contexts.

dPatj is required for pupal development

We did not observe any obvious morphological abnormalities, such as the loss of imaginal discs, in dPatj7/Df(3L)BSC123 (hereafter dPatj7/Df) larvae (data not shown). However, within several hours of pupation formation, the dPatj7/Df pupae exhibited deformities in shape, with a flattened anterior end and rounded posterior (Fig. 4A). At 16% pd, the head region was underdeveloped, although the imaginal discs appeared to have successfully everted (Fig. 4B), suggesting that even without dPatj the imaginal disc cells could still modulate their cell junctions to accomplish disc eversion (Pastor-Pareja et al., 2004). Older dPatj7/Df pupae failed to develop head and thoracic regions and growth of the everted imaginal discs stalled (Fig. 4C). The pupal defects and lethality were completely rescued in VK20-dPatj-L or dPatj7 VK20-dPatj7-GFP-S (Fig. 4D and supplementary material Table S1). The mild reduction in Crb expression observed in the dPatj mutants was unlikely to be responsible for the pupal lethality because the additional removal of one copy of crb in dPatj7/Df(3L)BSC123 crb76a or dPatj77 crb76a/dPatj7 did not enhance pupal lethality (supplementary material Table S1). The viability of dPatj7/two (Pielegre et al., 2003; Nam and Choi, 2006) suggests that the N-terminal 260 amino acids of dPatj might be sufficient for pupal development, but at present the specific function of dPatj in pupal development remains to be identified.

In summary, our molecularly defined dPatj null alleles conclusively show that dPatj is not essential for establishing or maintaining apical-basal polarity. The mild reduction in Crb expression in the absence of dPatj suggests that dPatj plays a supporting role in maintaining the Crb-Sdt complex in polarized epithelial cells. Such a scenario exhibits similarities with the situation for p120catenin (Adherens junction protein p120 – FlyBase) mutants: despite RNAi results suggesting that p120catenin is essential for cell adhesion, genetic mutants revealed that it only plays a supporting and non-essential role in cell adhesion in Drosophila (Myster et al., 2003). It remains possible, however, that mammalian Patj might have evolved increasingly important roles in apical-basal polarity, which might explain the differences between our dPatj null mutant phenotypes and those of mammalian cells with RNAi knocked-down Patj levels (Straight et al., 2004; Wang et al., 2004; Shin et al., 2005).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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