

**Planar cell polarity controls directional Notch signaling in the  
*Drosophila leg.***

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**Running title:**

PCP directs Notch signaling

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**Summary:**

The generation of functional structures during development requires tight spatial regulation of signaling pathways. Thus, in *Drosophila* legs, where Notch pathway activity is required to specify joints, only cells distal to ligand-producing cells are capable of responding. Here we show that the asymmetric distribution of planar cell polarity (PCP) proteins correlates with this spatial restriction of Notch activation. Frizzled and Dishevelled are enriched at distal sides of each cell and hence localize at the interface with ligand-expressing cells in the non-responding cells. Elimination of PCP gene function in cells proximal to ligand-expressing cells is sufficient to alleviate the repression, resulting in ectopic Notch activity and ectopic joint formation. Mutations that compromise a direct interaction between Dishevelled and Notch reduce the efficacy of repression. Likewise, increased Rab5 levels or dominant negative Deltex can suppress the ectopic joints. Together results suggest that PCP co-ordinates the spatial activity of Notch pathway by regulating endocytic trafficking of the receptor.

## INTRODUCTION.

The development and physiology of all multicellular organisms requires cell communication through well-defined signaling pathways that each consist of distinct canonical components. Cross-talk between pathways is required for the relatively limited number of pathways to match the anatomical and functional complexity that cell signaling has to regulate. The mechanisms that enable the pathway cross-talk are thus of major importance for generation and maintenance of complex structures.

One highly conserved signaling pathway important for coordinating many developmental processes is mediated by Notch transmembrane receptors (Fiuza and Arias, 2007; Fortini, 2009). Besides Notch (N), core members of this pathway in *Drosophila* include two transmembrane ligands, Serrate (Ser, Jagged in vertebrates) and Delta (DI). Upon ligand binding, N suffers two consecutive proteolytic cleavages and releases its cytoplasmic portion, which enters the nucleus and mediates a transcriptional response by binding to CSL transcription factors. Behind this apparent simplicity, a wide variety of biological functions and modes of action are made possible by context-dependent accessory mechanisms that help regulate the activation of N (Andersson et al., 2011; Bray, 2006). These include posttranslational modifications, such as glycosylation and ubiquitylation, that affect endocytic sorting of both N and its ligands.

N activity is also modulated by key aspects of tissue organization, including planar cell polarity (PCP). PCP was first characterized in *Drosophila* epithelial cells, where it establishes a polarity axis in the tissue plane, orthogonal to the apical-basal axis (Goodrich and Strutt, 2011; Vladar et al., 2009). Its relevance is evident in the orientation of cell projections, such as hairs or microvilli, and it is also important in

coordinating behavior in fields of cells, ensuring that they respond in a homogeneous directional fashion, including convergent extension in vertebrate embryos and ommatidial rotation in insect eyes. The latter is one example where PCP and N are known to converge (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999).

The proteins of one of the main PCP pathways (the core, Fz or Stan system) associate in complexes at the cell membrane. They include the transmembrane proteins Van Gogh/Strabismus (Vang/Stbm) (Taylor et al., 1998; Wolff and Rubin, 1998), Frizzled (Fz) (Vinson et al., 1989) and Flamingo/Starry Night (Fmi/Stan) (Chae et al., 1999; Usui et al., 1999) as well as the cytoplasmic proteins Prickle (Pk) (Gubb et al., 1999), Dishevelled (Dsh) (Klingensmith et al., 1994; Theisen et al., 1994) and Diego (Dgo) (Feiguin et al., 2001). In the wing epithelium, PCP protein complexes acquire an asymmetric proximal-distal localisation (Strutt and Strutt, 2009). A Fz-Dsh complex localises to the distal side of cells, together with Dgo, while a Stbm-Pk complex is localized to the proximal domain. These two complexes repel each other within the cell and both require Fmi and other proteins for their correct localisation. Most of these core PCP proteins function in other planar polarised systems in *Drosophila* and in vertebrates, although the details of their localisation or cellular actions may differ (Seifert and Mlodzik, 2007). Besides this role in PCP, a non-canonical Wnt pathway, Fz and Dsh are also required in canonical Wnt signalling, where they trigger nuclear accumulation of  $\beta$ -catenin upon Wnt activation (MacDonald et al., 2009). Most mutations in Fz affect its role in both PCP and Wnt signalling (Povelones et al., 2005), whereas PCP-specific mutations of Dsh exist and affect protein localisation (Axelrod et al., 1998). In addition, the interaction partners also influence the outcome since the

association with Dgo produces a bias towards PCP in detriment of Wnt (Wu et al., 2008).

A striking feature of flies mutant for core PCP members is that they have supernumerary joints in the tarsal region of the leg (Held et al., 1986). Normally composed of five segments (T1 to T5) separated by joints with a ball and socket structure, tarsi mutant for core PCP genes contain ectopic joints in segments T2, T3 and T4, and less frequently T1. Joints are determined at the end of larval development, when a stripe of Ser-expressing cells is specified within each segment and activates the receptor in distal cells triggering transcription of several N targets that control different aspects of joint differentiation (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). Ser appears to be the functional N-ligand in this process, since joints are absent in Ser mutants although other aspects of leg morphology appear normal, and in PCP mutant legs the ectopic joints correlate with ectopic Notch activity although the mechanism is unknown (Bishop et al., 1999).

The ectopic joint phenotype in PCP mutant flies implies that the PCP system has a role in regulating N signaling (Bishop et al., 1999). The likely scenario is that, when PCP is disrupted, N becomes activated in cells proximal to Ser-expressing cells, as well as those distal. How this regulation occurs is however unknown. In the eye, where the R3/R4 photoreceptor fate choice is critical for ommatidial polarity, Fz activity in the presumptive R3 is essential for polarizing N activity (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). It does so via a combination of mechanisms including effects on *Dl* transcription and activation as well as on endocytic regulation of N (Cho and Fischer, 2011; del Alamo and Mlodzik, 2006; Strutt et al., 2002) that may be amplified via Fmi up-regulation (Das et al., 2002). Whether these

mechanisms operate during other processes, such as joint development, remains to be established. Given the prevalence of PCP in many tissues, understanding how it can influence the ability of a cell to send or receive signals is of widespread relevance.

## MATERIALS AND METHODS

### *Fly stocks*

The following mutant alleles were used, either homozygous or in mitotic clones:  $pk^{sple1}$ ,  $dsh^1$ ,  $dsh^{v26}$ ,  $fmi^{192}$ ,  $fz^{J22}$ ,  $Drok^2$  (described in FlyBase, <http://flybase.org>). To monitor N activation we used the following reporter lines:  $bib-lacZ$ ,  $disco-lacZ$ ,  $E(spl)m\beta 1.5-CD2$  and  $E(spl)m\beta 1.5-lacZ$ . Sub-cellular localization of proteins was analyzed using GFP fusions:  $Ac5-Vang-GFP$  (Strutt et al., 2002),  $arm-fz-GFP$  (Strutt, 2001),  $dsh-GFP$  (Axelrod, 2001). For directed expression  $ap-Gal4$  or the flip-out cassette  $Ac>CD2>Gal4$  were used to drive the expression from UAS constructs:  $UAS-GFP$ ,  $UAS-dsh-myc$ ,  $UAS-Rab5$ ,  $UAS-Rab7$ ,  $UAS-Rab11$  (<http://flybase.org>),  $UAS-dx$ ,  $UAS-dx^{APRM}$ ,  $UAS-dx^{mRZF}$ ,  $UAS-dx^{ANBS}$  (Matsuno et al., 2002).

The FLP/FRT technique was used to generate mutant clones (Xu and Rubin, 1993) with appropriate recombinant chromosomes. To induce the FLPase, 48-72 h AEL (second instar) larvae were heat-shocked at 37°C in a water bath for one hour.

### *Histology, immunofluorescence, and microscopy*

Prepupal leg discs were dissected in PBS and fixed in 4% paraformaldehyde in PBS. Primary antibodies were: rabbit  $\alpha$ - $\beta$ -galactosidase (Life Technologies, Grand Island, NY), mouse  $\alpha$ - $\beta$ -galactosidase (Promega, Madison, WI), rabbit  $\alpha$ -GFP (Rockland, Gilbertsville, PA), mouse  $\alpha$ -CD2 (AbD Serotec, Kidlington, UK), rabbit  $\alpha$ -Serrate (gift of Ken Irvine, Waksman Institute of Microbiology, NJ), rabbit anti-Rab5 (Abcam, Cambridge, UK) mouse  $\alpha$ -Arm and mouse  $\alpha$ -Fmi (both from Developmental Studies Hybridoma Bank, University of Iowa). For detection we used fluorophore-conjugated

secondary antibodies (Jackson Immunoresearch Laboratories Inc, West Grove, PA). Actin cytoskeleton was labelled with Phalloidin-TRITC (Sigma, St. Louis, MO).

Samples were examined using a Leica DM RXA2 microscope and Leica TCS SP Confocal system (Leica Microsystems, Wetzlar, Germany). Images were processed and analyzed with the Leica Confocal Software, the ImageJ suite, and Adobe Photoshop.

### ***dsh* genomic constructs.**

Specific mutations were introduced into the coding sequence of the *dsh-GFP* genomic fragment (Axelrod, 2001) using site directed mutagenesis. In brief, a 1.4kb fragment containing part of the promoter and the coding region for the DIX and PDZ domains was subcloned into pKS for mutagenesis using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) to introduce the K46V and Q47A mutations. The mutated region was then substituted into the full length *dsh-GFP*, by replacing the 400bp *MluI-KpnI* fragment encompassing the DIX domain. The entire *dsh-GFP* mutant genomic fragment was introduced into the transformation vector pWhiteRabbit. Single copy insertions of the resulting plasmid were generated using conventional P-element mediated transformation and multiple insertion lines were mapped to chromosomes and analyzed for expression. Suitable lines were then crossed into *dsh<sup>1</sup>* and *dsh<sup>v26</sup>* backgrounds to generate  $w^{114} dsh^{[x]} / FM7$ ;  $dsh^{mut6} (w^+) / dsh^{mut6} (w^+)$  and the phenotypes analyzed in  $dsh^{[x]} / Y$  males. Over 100 legs were scored for ectopic joint phenotype for each one of the independent constructs.

### ***Biochemistry***



To map the domains of Dsh interacting with NICD, GST pull-down experiments were performed as described (Djiane et al., 2005); by incubating GST-NICD fusion with <sup>35</sup>S-labeled Dsh fragments (details of primers used available on request). Specific amino acids were mutated by site directed mutagenesis with the QuikChange kit (Agilent Technologies, Santa Clara, CA). The relative intensity of the bands was calculated using the gel analysis application of the ImageJ suite, by plotting of the lane profile and calculation of the resulting peak areas. Two gels were analysed for each pair of bands.

## RESULTS

### ***The double joint planar cell polarity phenotype correlates with ectopic N activity.***

Mutations affecting core PCP genes result in supernumerary joints in the tarsal region, most commonly in tarsal segments 2-4 (Bishop et al., 1999; Held et al., 1986). Ectopic joints are located proximal to the normal joint and have reversed polarity (Fig. 1A, B). Such ectopic joints appear to be a *bona fide* PCP phenotype, as the defect occurs with mutations affecting any core PCP gene: phenotypes of *pk*, *fz*, *dsh*, *Vang*, *fmi*, and *dgo* are consistent and differ from other mutant conditions affecting joint development (Held et al., 1986; Wolff and Rubin, 1998) (<http://flybase.org> and our unpublished data).

The implication of this phenotype is that PCP controls the directionality of cell signaling mediated by the N pathway, the main agent in joint determination (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). To ascertain whether this interpretation is correct, we have analyzed expression of three reporters of N activation in different viable mutants of core PCP genes. Reporter expression is fully established by 2-6 hours after puparium formation (APF) when the leg disc starts to evert. The best characterized direct N targets are the *E(spl)* genes. An *E(spl)mβ1.5-lacZ* reporter has previously been shown to respond to N in the leg (Cooper et al., 2000; de Celis Ibeas and Bray, 2003) where it is expressed in a stripe distal to, and partially overlapping, the Ser-expressing cells. In prepupal legs of *dsh<sup>1</sup>*, a PCP specific allele, an ectopic domain appeared, confirming that disruptions in core PCP gene activity result in ectopic N activity (Fig. 1C, D). Equivalent ectopic domains of *E(spl)mβ1.5-lacZ* were seen with alleles affecting other core PCP genes (see below).

Similar results were obtained with a *lac-Z* insertion into *big brain* (*bib-lacZ*), whose expression is also regulated by N (de Celis et al., 1998; Pueyo and Couso, 2011). *bib-lacZ* is expressed in a narrow band one or two cells wide just distal to the domain of Ser expression (Fig. 1E, I). In prepupal legs of *pk<sup>sple1</sup>*, a strong hypomorphic allele of *pk* (Gubb et al., 1999) there was an ectopic *bib-lacZ* stripe proximal to the Ser-expressing cells (Fig. 1F, J). In addition, the levels of *bib-lacZ* were sometimes reduced or discontinuous (see also below). A further marker of leg joints (although not a known direct target of N) is *disco-lacZ* (Bishop et al., 1999), an insertion into *disconnected*, which is expressed in a broader domain spanning three or four cell diameters (Fig. 1G). In a *pk<sup>sple1</sup>* mutant, the *disco-lacZ* domain was duplicated and, due to its larger territory, the ectopic domain merged with the endogenous one from the preceding segment resulting in a continuous domain of expression in most of the tarsal region (Fig 1H).

Despite the evidence for ectopic N activity, there was no change in expression of the Ser ligand in *pk<sup>sple1</sup>* legs or in mitotic clones of *fz<sup>J22</sup>* (a strong *fz* hypomorph; Fig. 1I-K) or of *Dl* present at later stages (Bishop et al., 1999). Neither was there a clear alteration in the expression-profile of the N receptor itself (Fig. S1). Therefore, the extra stripes of N activation are a general feature of mutations affecting PCP but are unlikely the result of simple change in the expression of N or its ligands.

***Cell autonomous effects of PCP alleles suggest a requirement in the signal-receiving cell.***

In order to know whether the mechanism of action of PCP on N is direct or indirect, or whether it is likely to act in ligand sending or signal receiving cells, we analyzed defects

caused by clones of mutant cells. If the effects are directly in signal-receiving cells, activation of N targets should only be detected autonomously within mutant cells in clones located proximal to the site of ligand expression. Conversely, if PCP regulation affects the ligand some non-autonomous defects would be seen. It is important to note that not all mutant alleles would be useful for this analysis, since several of them show a directional domineering non-autonomy due to reorganization in PCP over neighboring cells. Therefore, we used only alleles reported to show autonomous polarization phenotypes: *pk<sup>sple1</sup>*, *fmi<sup>192</sup>*, *dsh<sup>1</sup>*, and *fz<sup>J22</sup>* (Chae et al., 1999; Jones et al., 1996; Lee and Adler, 2002; Strutt and Strutt, 2007).

We first examined effects on the *E(spl)mβ1.5-CD2* reporter (containing the same regulatory element as *E(spl)mβ1.5-lacZ*) a direct target of N pathway. In clones of *fz<sup>J22</sup>*, expression of *E(spl)mβ1.5-CD2* is de-repressed autonomously within the mutant cells (Fig. 2A). Furthermore, in several examples the mutant cells were juxtaposed with putative ligand producing cells that were wild type. These results argue that the effect of *fz<sup>J22</sup>* on N pathway is autonomous and is most likely to occur in the signal-receiving cells. *E(spl)mβ1.5-CD2* is also autonomously de-repressed in *dsh<sup>1</sup>* mutant clones (not shown).

The behavior of *disco-lacZ* in *pk<sup>sple1</sup>* and *fmi<sup>192</sup>* clones was identical to that of *E(spl)mβ1.5-CD2*. In both genotypes *disco-lacZ* was de-repressed in a completely autonomous manner (Fig. 2B, C). Again, expression of *lacZ* in cells at clone edges suggested a requirement for polarization in the signal receiving cell rather than on the ligand.

The effects of mutations on *bib-lacZ* were however slightly different. In all 3 of the mutants tested (*dsh<sup>1</sup>*, *pk<sup>sple1</sup>*, *fmi<sup>192</sup>*) de-repression of the reporter was detected only in

mutant cells and not in adjacent wild-type cells (Fig. 2D, E and Fig. S2). However, unlike *E(spl)mβ1.5-CD2* and *disco-lacZ*, ectopic activation of *bib-lacZ* was variable in intensity (weak to similar to endogenous *bib-lacZ*) and in extent. In some clones only a few cells showed ectopic expression while in others it filled the whole width of the mutant clone. Nevertheless, the fact that *bib-lacZ* can be de-repressed at the clone boundaries is consistent with a requirement for PCP in signal receiving cells, rather than through effects on Ser. We note also in some *fmi* and *pk* clones the endogenous domain of *bib-lacZ* is weakened within the clone (Fig. 2E). This observation also correlates with the fact that in whole legs mutant for core PCP genes, normal *bib-lacZ* expression can be weakened compared to the ectopic (Fig 1F and Fig. S2).

Both the variability of *bib-lacZ* de-repression within clones and the weakening of endogenous expression in mutant cells may reflect a difference in the threshold of N activity required for *bib-lacZ* activation compared to *disco-lacZ* and *E(spl)mβ1.5-CD2* (see Discussion). Nevertheless, N reporters were de-repressed autonomously in all the core PCP mutant genotypes when mutant cells were located proximal to the Ser domain. No ectopic expression of the reporters was observed in wild type tissue adjacent to the mutant cells, which argues against an effect of PCP on Ser or on a second signaling pathway. These results were replicated in earlier stages (third instar leg discs) and in smaller clones (Fig. S3).

### ***Asymmetrical distribution of core PCP proteins in the developing leg***

Core PCP proteins adopt a polarized distribution in *Drosophila* pupal wing cells, which are arranged in a regular hexagonal lattice (between 20 and 24 h APF). Dsh and Fz

become localized to the distal edge of each cell, Pk and Vang to the proximal edge (Strutt and Strutt, 2009). Some degree of polarization is also evident in prepupal stages (Aigouy et al., 2010; Strutt et al., 2011). Cells in the prepupal leg epithelium are mostly irregular in shape and size, unlike the wing, making it difficult to detect clear organization in cell morphology or protein distribution. To investigate whether there was any asymmetry in core PCP protein distribution, we generated patches of cells expressing Fz::GFP, Vang::GFP and Dsh::GFP and examined protein distributions at clone borders, as was done previously in the wing to investigate protein asymmetries (Axelrod, 2001; Strutt et al., 2002; Strutt, 2001). Fz::GFP and Dsh::GFP levels were higher at the distal side of cells compared to proximal (Fig. 3A, B). Conversely, Vang::GFP levels were highest on the proximal sides of each cell (Fig. 3C). Some differences in Fmi localization were also evident: the protein was more enriched at proximal-distal boundaries than at dorsal-ventral (Fig. 3D), a characteristic that was most obvious in the tarsus-pretarsus boundary where cells have a more regular morphology (Fig. 3D').

Cells receiving the N signal also exhibited distinct morphology. Detection of  $\beta$ -catenin, localized to sub-apical adherens junctions, revealed that the *bib-lacZ*-expressing cells were roughly quadrangular. Their distal edges formed a straight line, most likely marking the boundary between adjacent tarsal segments (Fig. 3E). These features were also observed in the ectopic domain of *bib-lacZ* in the PCP mutants (Fig. 3F). In addition, the intensity of *bib-lacZ* expression was correlated with cell morphology, both in the normal and ectopic domains of expression. This suggests that high levels of N activation result in ordered alignment of the leg epithelial cells.

### ***Direct interaction of N and Dsh.***

Drok is one of the main mediators of the cytoskeletal response to PCP: it is important for restricting wing hair generation and for ommatidial rotation (Winter et al., 2001). We tested whether Drok had any effect on the *bib-lacZ* and *E(spl)mβ1.5-CD2* reporters. Although *Drok* mutant clones showed defects in ommatidial rotation and in tissue morphology (data not shown), none resulted in ectopic expression of the *bib-lacZ* or *E(spl)mβ1.5-CD2* reporters (Fig. 4A, B). Therefore, this function of the core PCP pathway does not seem to be mediated by the actin cytoskeleton.

Previous studies have shown a physical interaction between Dsh and N, which contributes to inhibition of N signaling in the wing margin (Axelrod et al., 1996; Munoz-Descalzo et al., 2010). We questioned whether a similar mechanism operates in joint regulation. Overexpression of Dsh driven by *ap-Gal4* in T4 and proximal T5 had two clear effects (Fig. 4C, D). First, there was a disruption of PCP already described for Dsh overexpression in the wing (Axelrod et al., 1998). Second, formation of the joint between these tarsi was repressed, as would be expected if Dsh was capable of repressing N. A possible caveat to this interpretation is that Dsh overexpression could produce patterning defects causing a secondary effect on joints. However, the effects from expressing Dsh in clones do not support this possibility. For example, a large dorsal clone of Dsh-expressing cells produced autonomous repression of *bib-lacZ* (Fig 4E, E'). Although this clone contained two small putative axis duplications (ectopic leg tips in the form of circular domains of *bib-lacZ* expression in tarsi 2 and 5, arrowhead in Fig. 4C'), segmentation was largely unaltered.

To test functional relevance of the direct interaction of Dsh and N in leg segmentation we set out to find a mutant form of Dsh that had reduced ability to physically interact

with N. To map the interacting regions we used glutathione-S-transferase (GST) pull-down experiments. From a set of deletions spanning different regions of Dsh, only constructs with an intact DIX domain were successfully retained by the intracellular portion of N (GST-NIC, Fig. 5A, B). Deletion of the N-terminal region of this DIX domain was sufficient to abolish this interaction (compare constructs Dsh5 and Dsh6). Unfortunately, as the DIX domain is also required for Axin recruitment (Julius et al., 2000; Kishida et al., 1999), these deletions also prevented Axin binding (Fig. 5B). It was therefore important to identify mutations that would affect binding of NIC but not Axin. We first tested effects of mutations in residues that are conserved between Dsh and the mammalian Dvl proteins. Of the 3 mutations tested, only V43E present in mut1 and mut4 abolished interaction with NIC and this also affected Axin binding (Fig. 5C-E and data not shown). Substitution of two adjacent hydrophilic residues that are specific of *Drosophila* Dsh, K46V+Q47A, showed some specificity for NIC. Quantitative analysis of the band intensities revealed that the mutation (*mut6*) reduced binding to NIC by 95 %, but interaction with Axin was reduced only 65%. This mutation was therefore a candidate to test relevance for N regulation.

To investigate whether the *mut6* form of Dsh was compromised for N regulation in the leg, we introduced the K46V+Q47A mutation into a *dsh* genomic rescue construct that had been used previously (Axelrod, 2001). Multiple insertions of the mutant protein were tested for their ability to rescue *dsh*<sup>1</sup> and *dsh*<sup>v26</sup> mutant phenotype. The former only affects PCP function; the latter is a null allele affecting both PCP and Wnt signaling. All three constructs could rescue the embryonic lethality of *dsh*<sup>v26</sup>, so they can function in the canonical Wnt pathway. As expected, the PCP ectopic leg joint phenotype was rescued by wild type *dsh* construct but not by *dsh*<sup>1</sup> (Fig6A). Importantly the *mut6*



constructs showed a reduced ability to rescue the ectopic leg joint phenotype (Fig 6A, B), although the proximal-distal and dorsal-ventral patterns were wild type, including the distal tip, which is the most sensitive part of the leg for alterations in Wnt signaling (Galindo et al., 2002). In addition, when combined with *dsh*<sup>v26</sup>, the resulting adult flies had largely wild-type wings and both leg bristles and wing hairs exhibited normal planar polarization (Fig 6B-E). Expression levels of the GFP-tagged Dsh proteins produced by *mut6* were similar to that from wild-type *dsh* construct (Fig- 6B, C). These results therefore are consistent with the hypothesis that a direct interaction between Dsh and N is important for suppressing N activity in the domain proximal to Ser stripe. However, we cannot fully rule out the alternate possibility that the inability of *mut6* to rescue leg joints reflects a difference in the threshold levels of Dsh activity required for this process compared to others.

Antagonistic effect of Dsh on N signaling has been described previously in the wing, where N is also important for patterning sensory organs at the wing margin. However, although overexpression of known N repressors such as Numb (Nb) and Hairless (H) (Frise et al., 1996; Nagel et al., 2005) produced nicks in the wing margin and repression of the Notch-target Cut (Fig S4), ectopic Dsh was neither sufficient to produce nicks in the adult wing nor to repress Cut at larval stages (Fig. S4). Although the interpretation of these results may be confounded by the fact that overexpression of Dsh at the margin can also lead to expression of N ligands, and N activation, possibly explaining the ectopic bristles observed in the wing blade (Fig S4), the differences with the wild type wing are nevertheless relatively minor compared to the overexpression of Nb and H. The results suggest therefore that the ability of Dsh to suppress N is restricted to certain contexts, making it less likely that it antagonizes the cleaved, active form of N (N<sub>icd</sub>).

To further test whether Dsh could antagonize Nicd, we assayed the consequences on a N responsive reporter (NRE-luciferase) of co-expressing Dsh and Nicd in transient transfection assays. Expression of Nicd alone resulted in strong induction of NRE-luciferase that was little altered by co-expression with Dsh (Fig. S4). Therefore, although Dsh binds to the intracellular domain of N, it does not inhibit Nicd trans-activation function, suggesting it is likely to regulate the receptor prior to cleavage.

### ***Role of endocytic regulators***

It has been suggested that Dsh influences endocytic trafficking of proteins including N (Chen et al., 2003; Munoz-Descalzo et al., 2010; Yu et al., 2007). One model therefore is that recruitment of Dsh to the distal edge of the cells would result in down-regulation of N by endocytosis. To investigate this we tested the consequences of expressing several different endocytic regulators in the T4 segment with *ap-Gal4* (Fig 4C) to determine their effect on the ectopic joint present in *dsh<sup>1</sup>* mutants.

First we tested consequences of expressing 3 different Rab GTPases, Rab5, Rab7 and Rab11, which regulate vesicle trafficking to early endosome, late endosome and recycling endosome compartments respectively (Stenmark, 2009). In previous studies, overexpression of Rab5-GFP and Rab7-GFP were able to suppress the ectopic N activation seen in *lethal giant discs (lgd)* mutants (Jaekel and Klein, 2006). Overexpression of Rab5 in a wild type background had no effect (Fig. 7A, C), but in a *dsh<sup>1</sup>* mutant background it was able to modify the ectopic joint phenotype in 80% of the legs examined, resulting in a partial suppression (Fig. 7B, D). In contrast neither Rab11 nor Rab7 had any effect in this assay (data not shown) suggesting that the defect is linked to

transit to early endosomes. The implication is that *dsh*<sup>1</sup> leads to a defect in endocytic transport of N from the plasma membrane preventing its activation by Ser, and that this can be compensated by increasing the levels of Rab5. To test this hypothesis, we examined whether over-expression of Dsh::myc, which resulted in lack of joints and down-regulation of bib-lacZ (Figure 4C-E), had any impact on N protein distribution. In *ap-Gal4, UAS-dsh-myc* legs there was reduced N at the apical membrane and a large fraction of N co-localised with Dsh in intracellular puncta (Fig. 8A). Many of the N-containing puncta appeared to correspond to early endosomes based on their co-localization with Rab5 (Fig 8B). Furthermore, N depletion from the cell surface and accumulation in puncta was evident using antibodies against either the extracellular or the intracellular portion of N (Figure 8 C, D). This implies that a significant fraction of the endocytosed N is uncleaved and therefore that the Dsh-mediated change in localization occurs independent of ligand binding or  $\gamma$ -secretase cleavage.

Rab5 has also been found to inhibit the ectopic N signaling caused by increased levels of the Deltex (Dx) E3 ubiquitin ligase (Hori et al., 2004; Matsuno et al., 2002). The effect of Dx is complex, since it can result in ligand-independent activation or in downregulation of N signaling depending on the context (Mukherjee et al., 2005; Wilkin et al., 2008; Yamada et al., 2011). Both modes of regulation require Rab5-mediated endocytosis of N to early endosomes. To test whether the *dsh*<sup>1</sup> phenotype in T4 could be modified by expression of Dx and derivatives, we assayed full length Dx and mutations affecting some of its functional domains (Matsuno et al., 2002): proline-rich region (Dx <sup>$\Delta$ pro</sup>), ring-H2 domain (Dx<sup>mRZF</sup>) and N-binding region (Dx <sup>$\Delta$ NBS</sup>).

Expression of Dx and Dx <sup>$\Delta$ pro</sup> phenocopied the defects caused by PCP mutations (Fig. 7E), arguing that N activity at these ectopic sites may involve modifications to its

trafficking, and neither was able to modify the *dsh<sup>l</sup>* phenotype (Fig. 7F).  $Dx^{mRZF}$  had no effect in wild type or in *dsh<sup>l</sup>* (not shown). In contrast, expression of  $Dx^{\Delta NBS}$  resulted in a striking phenotype of joint fusion (Fig. 7 G, H), in both wild-type and *dsh<sup>l</sup>*, resembling consequences of *dx* null alleles in certain conditions of altered N activity (Gorman and Girton, 1992).  $Dx^{\Delta NBS}$  thus appears to have a dominant negative effect, blocking the endogenous T4-T5 joint as well as the ectopic joint in *dsh<sup>l</sup>*. This contrasts with the wing, where  $Dx^{\Delta NBS}$  exhibits little residual activity (Matsuno et al., 2002) and suggests that the Dx context-dependent effects (Wilkin et al., 2008), are likely to rely on other factors which could be titrated by  $Dx^{\Delta NBS}$ .

Our results reveal that two endocytic regulators, Rab5 and Dx, can alter N activation at the site of ectopic joint formation, although there are mechanistic differences between them. Over-expression of Rab5 had no effect on wild type tarsus but could rescue the mutant phenotype of *dsh<sup>l</sup>*. This suggests that ectopic N activity in PCP mutants is associated with a change in N trafficking that can be suppressed by Rab5. In contrast, over-expression of Dx induced ectopic joints even in the wild type background arguing that it is sufficient to overcome repression of N mediated by PCP.

## DISCUSSION

Spatially co-ordinated regulation of signaling pathways is essential to generate correct anatomical and functional structures, as exemplified by the *Drosophila* leg where activity of the N pathway is required to specify leg joints (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). In this case, only cells distal to the stripe of Ser expression appear capable of responding to the ligand. Here we show that activity of the core PCP pathway is required in those cells proximal to the domain of Ser expression to prevent them from responding to this N ligand. This regulation correlates with the asymmetric distribution of the core PCP proteins, as we show that Fz/Dsh are enriched at the distal side of each cell, which in the non-responding cells faces the neighboring Ser-expressing cells. Conversely in those cells distal to Ser, Fz/Dsh are depleted from the proximal side, leaving N free to interact with its ligand to promote joint formation. It appears that elimination of core PCP gene function in cells proximal to the Ser-expressing cells, is sufficient to alleviate the repression resulting in ectopic N activity and ectopic joint formation. Such regulation of the membrane availability of Notch could equally affect Dl mediated activation, although Ser appears the major ligand responsible in the joints (Bishop et al., 1999). Other factors are likely to influence proximal repression of N since ectopic joints are also observed in alterations of the EGFR pathway (Galindo et al., 2005) and mutants of *defective proventriculus* (Shirai et al., 2007).

We note also that the domains of N activation (both normal and ectopic) extend beyond the cells at the interface with Ser. We have not sought to investigate this additional level of regulation here, but our results indicate that it is unlikely due to a secondary signal emanating from the Ser-interfacing cells because the loss of function clones show

complete autonomy, without any “shadow” of activation adjacent to the clone. An alternative possibility is that the cells make more extensive contacts, as has been seen in other tissues (Cohen et al., 2010; De Jossineau et al., 2003; Demontis and Dahmann, 2007).

PCP regulation of N has been observed in other developmental processes, most notably in photoreceptor fate choice in the *Drosophila* eye (Cooper and Bray, 1999; del Alamo and Mlodzik, 2006; Fanto and Mlodzik, 1999; Strutt et al., 2002). There, much of the regulation is via effects on levels and activity of the ligand. However, we detected no change in the pattern of N or Ser expression in PCP mutants. Instead our evidence suggests that regulation involves direct interaction between Dsh and N and that this interaction has consequences on the endocytic trafficking of N, resulting in its inactivation. The interaction requires the amino-terminal portion of the Dsh DIX domain, which is also required for Axin-binding in the canonical Wnt pathway (Julius et al., 2000; Kishida et al., 1999), making it difficult to dissect its role in the PCP-mediated N inhibition. Nevertheless, we were able to generate one mutation that reduced interactions with N with minor consequences on Axin binding. Rescue experiments with this mutant form of Dsh indicated that it was less effective in PCP function in the leg joints compared to others (e.g. polarity of leg bristles). These results support the model that a direct interaction between Dsh and N is relevant in the context of joint determination. However, we cannot fully rule out the possibility that the mutation has more generalized effects on Dsh, if the joints are particularly sensitive to the levels of Dsh activity.

Several studies indicate that endocytic sorting of N is involved in its regulation, with either positive or negative effects depending on the particular context (Fortini and

Bilder, 2009; Furthauer and Gonzalez-Gaitan, 2009). Our findings suggest that regulation of N by PCP in the leg is mediated by interaction with Dsh, and probably involves the control of N endocytic trafficking. This suggests a model whereby the interaction between Dsh and N results in increased endocytosis of the N receptor, so reducing its capability to interact with ligands on neighbouring cell. Removal of Fz or Dsh compromises this endocytic trafficking, allowing N to be activated. The interaction between Dsh and N is thus only likely to be relevant under circumstances where there is a strong localization of Dsh co-incident with an interface between N and ligand expressing cells.

Previous studies have also suggested a role for Dsh in regulating N and on promoting its endocytosis (Axelrod et al., 1996; Munoz-Descalzo et al., 2010). In both instances these effects were linked to Wg signaling, rather than core PCP pathway as here. Nevertheless several aspects are consistent with our results, most notably the direct binding between Dsh and N. Additionally it has been argued that Dsh specifically antagonizes Dx mediated effects of N (Romain et al., 2001), which is compatible with their complementary effects on joint formation. However, it is also evident that the ability of Dsh to inhibit N depends on the developmental context. For example, while over expression of Dsh in the leg is sufficient to inhibit N activation at presumptive joints, overexpression of Dsh at the wing margin is not sufficient to repress N signaling: there are no nicks and *cut* expression is not inhibited. Interestingly, differences in Dx behaviour are also evident in these two contexts. At the wing margin (Matsuno et al., 2002),  $Dx^{\Delta pro}$  acts as a dominant negative form of Dx, while  $Dx^{\Delta NBS}$  is inactive. In contrast, in the leg joints  $Dx^{\Delta pro}$  behaves as wild type Dx, while  $Dx^{\Delta NBS}$  is a dominant

negative. We postulate therefore that the subcellular localization of Dsh and the availability of Dx are important for determining the regulation of N trafficking at joints.

The autonomous effect of core PCP mutants was clear when we used the *E(spl)mβ1.5-CD2* N reporter and *disco-lacZ*. However, the consequences on *bib-lacZ* were more complex. Although larger clones of mutant cells always exhibited autonomous ectopic expression, similar to *E(spl)mβ1.5-CD2*, some narrow clones exhibited no ectopic expression. We suggest that this may be due to *bib-lacZ* having a higher threshold of response, so it would need stronger N activation. The domain of *bib-lacZ* is narrower than that of the other known reporters in agreement with this model. Furthermore, we found some cases where there was a reduction of the normal *bib-lacZ* expression in the mutant cells, in addition to ectopic expression. This suggests that PCP mediated distal localization of Dsh would be required not only for inhibition of N in proximal cells, but also for efficient activation of N in distal ones.



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## FIGURE LEGENDS.

**Figure 1. *N* is ectopically activated in PCP mutants.**

(A) Wild type leg with normal joints (arrowhead) and (B)  $pk^{sple1}$  leg with ectopic joints with inverted polarity. In these and subsequent panels arrowheads indicate normal joints terminating tarsal segments 2, 3 and 4, and the corresponding domains of expression. (C, D)  $E(spl)m\beta 1.5-lacZ$  expression (X-gal, blue) in wild type prepupal leg (C) and  $dsh^1$  mutant (D) with ectopic stripes. (E, F)  $bib-lacZ$  expression (green) in a single row of cells (anti- $\beta$ -Catenin, purple, cell contours) at the segment boundary in wild-type (E) is duplicated in a  $pk^{sple1}$  (F). (G, H)  $disco-lacZ$  (green) is expressed in a wider domain in wild-type (G), normal and duplicated domains merge in a single broad territory in  $pk^{sple1}$  (H). (I)  $bib-lacZ$  (green) is expressed distally adjacent to Ser (purple), and (J) in  $pk^{sple1}$  the ectopic stripe appears proximally adjacent to Ser; a sub-apical confocal section is shown to capture the nuclear  $\beta$ -galactosidase. (K) Large  $fz^{J22}$  clone marked by absence of GFP (green); expression of Ser (purple) is not altered.

**Figure 2. Proximal de-repression of *N* in PCP mutants is cell-autonomous.**

In all panels mutant clones are revealed by absence of GFP (green); ectopic expression of reporters (purple, single channel on the right) is indicated by arrowheads. (A) Mutant  $fz^{J22}$  clone; ectopic expression of  $E(spl)m\beta 1.5-CD2$  coincides with clone border despite presence of adjacent wild type (ligand-expressing) cells. (B) Mutant  $fmi^{192}$  clones, autonomous expression of  $disco-lacZ$  in cells adjacent to wild-type GFP-positive cells. (C) Mutant clones of  $pk^{sple1}$ , several ectopic domains of  $disco-lacZ$  occur autonomously within the mutant clones. (D) Large clone of cells mutant for  $dsh^1$ , some ectopic

expression of *bib-lacZ* appears in tarsal segment 3, but not in segment 2. (E) Elongated clones of cells mutant for *pk<sup>sple1</sup>*, ectopic activation of *bib-lacZ* that can occupy the full width of the clone. Occasional down-regulation of the normal stripe of *bib-lacZ* is also detected (arrows).

**Figure 3. Cell biology of PCP in the prepupal leg.**

(A-D') Localization of PCP proteins, proximal-distal orientation is top left to bottom right; cells outlined with anti-E-cadherin (A-C, purple) (A) Cells at the border of Fz::GFP-expressing clones reveal that the fusion protein is enriched at the distal side (arrowhead, compare with arrow marking proximal cell boundaries); (B) Expression of Dsh::GFP is comparatively weak, but also accumulates distally (arrowhead). (C) Vang::GFP localizes to the proximal side of cells (arrow). (D, D') Fmi is absent from cell borders oriented along the dorsal-ventral axis in t2 (D) and in the tarsus-pretarsus boundary (D'). (E) Late prepupal leg, cells expressing *bib-lacZ* (green, dotted) have a larger sub-apical diameter ( $\beta$ -catenin, purple; single channel on the right), more regular shape and their borders align to form a straight line. (F) Late prepupal *pk<sup>sple1</sup>* leg, ectopic rows of *bib-lacZ* expression have the same features; intensity of *bib-lacZ* appears to correlate with cell size, shape and alignment both in normal and ectopic domains.

**Figure 4. N down-regulation by Dsh.**

(A, A') Clone of cells homozygous for *Drok<sup>2</sup>* (marked by the absence of GFP, green), no ectopic expression of *bib-lacZ* is detected (purple, single channel in A'). (B, B') In clones of the same genotype expression of *E(spl)m $\beta$ 1.5-CD2* is also unaffected. (C) Ap-

Gal4 expression domain revealed with UAS-GFP (green), includes t4 and proximal part of t5 (anti-E-cadherin, purple). (D) *ap-Gal4*, *UAS-dsh* causes defects in planar polarity and the joint between t4 and t5 is absent (brackets indicate *ap-Gal4* territory). (E, E') Large dorsal clone expressing Dsh::myc (anti-myc, green) results in autonomous repression of *bib-lacZ* (purple, separate channel in E'), and putative axis duplications (ectopic leg tips, arrowheads). All tarsal segments are present with segmental grooves detected as normal.

**Figure 5. Direct interaction of Dsh and N.**

(A) Domain structure of Dsh protein, with the different deletion constructs employed in the GST pull-down assays depicted below: constructs retained by GST::NIC are ticked. (B) Autoradiograph of <sup>35</sup>S labeled Dsh constructs pulled down with GST::NIC and GST::Axin. Input proteins and a negative control pull-down using an unrelated GST construct (-) are also shown. (C) Alignment of the N- and Axin-interacting region of *Drosophila* Dsh with two human Dishevelled (Dvl) proteins and *Drosophila* and human Axin. Residues mutated are in bold: D39 and V43 are conserved in all Dsh proteins but not in Axins, and K46 and Q47 are present only in *Drosophila* Dsh. (D) Six different site-directed mutants were generated with single or double mutations. (E) GST pull-down assays with the different <sup>35</sup>S labeled Dsh mutants. V43E prevents interaction with NIC and Axin, K46V + Q47A (mut6) hinders the interaction with NIC but retains some interaction with Axin. As a control, GST::Grh fails to bind to any Dsh derivatives.

**Figure 6. Rescue with genomic constructs.**

(A) Presence of ectopic joints in tarsal segments 2,3 and 4 in  $dsh^1$  and  $dsh^{v26}$  mutant backgrounds rescued with genomic constructs encoding different forms of  $dsh$ :  $dsh^1$  (1 insertion) wild type  $dsh$  (5 different insertions), and  $dsh^{mut6}$  (3 insertions);  $yw$  is used as a wild type control. (B, C) Representative legs of  $dsh^{v26}$  rescued with the genomic constructs for wild type  $dsh$  (B) and for  $dsh^{mut6}$  (C), arrowheads point to partial ectopic joints, the insets show similar expression levels of Dsh::GFP and Dsh<sup>mut6</sup>::GFP in leg imaginal discs. (D, E) Wings of  $dsh^{v26}$ ;  $dsh^{mut6}$  (D, trichomes are *forked*) and  $dsh^1$ ;  $dsh^{mut6}$  (E) rescued flies with largely normal wing margin and PCP.

**Figure 7. Endocytic control of N signaling in the leg.**

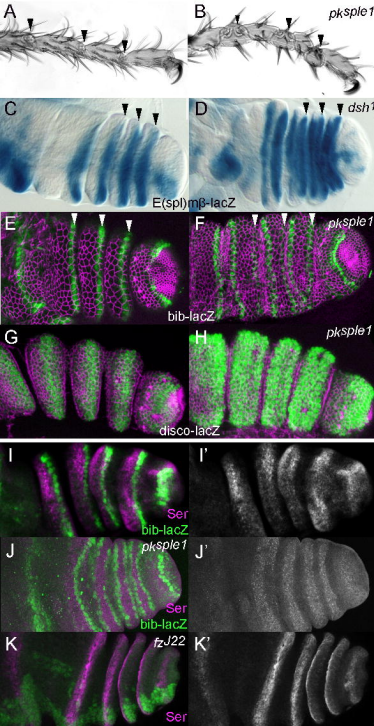
Endogenous (black) or ectopic (white) joints in the tarsus 4/5 region detected in wild type (A,C,E,G) and  $dsh^1$  (B,D,F,H) backgrounds, after expression of different endocytic regulators driven by *ap-Gal4*. Arrows are complete joints, arrowheads partial joints (A,B) Normal and ectopic joints in t4/t5 region. (C,D) Expression of Rab5 has no effect on wild type (C) but partially suppresses ectopic joints in  $dsh^1$  (D, white arrowhead; note only legs with ectopic t3 joint were scored for rescue of ectopic t4 joint). (E,F) Expression of Dx elicits ectopic joint in wild type resembling  $dsh^1$  (E) and fails to modify  $dsh^1$  (F). In contrast, expression of Dx<sup>NBS</sup> partially inhibits normal joints in wild type (G) and partially suppresses normal and ectopic joint in  $dsh^1$  (H).

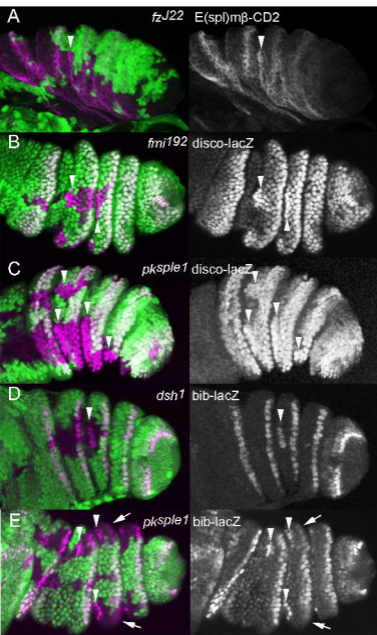
**Figure 8. Ectopic expression of dsh-myc in the ap territory.**

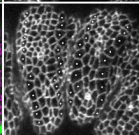
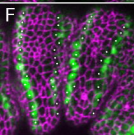
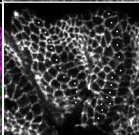
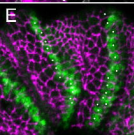
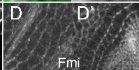
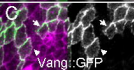
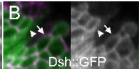
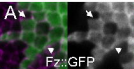
(A) Over-expressed Dsh::myc (anti-myc, purple) appears in a vesicular pattern and co-localises with N (anti-Necd, green). (B) Some N vesicles (Necd, green), coincide with

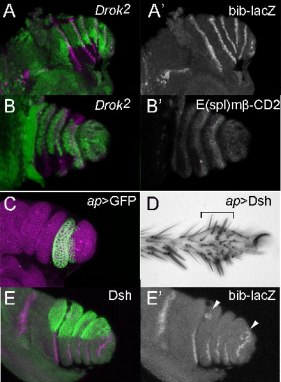


Rab5-positive puncta (anti-Rab5, purple; e.g. arrows). (C,D) Sub-cellular localization of N (green) is altered in Dsh-expressing cells (purple). Immunofluorescence associated with apical cell membrane is decreased, internal puncta are increased. Similar results are obtained with antibodies against extracellular (C) or intracellular (D) portions of N.









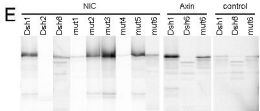
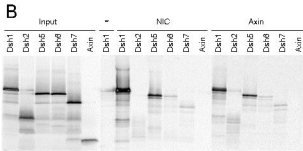


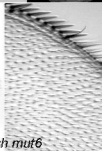
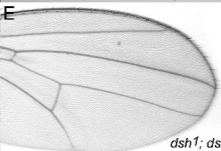
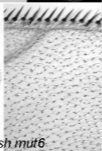
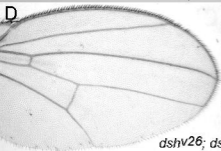
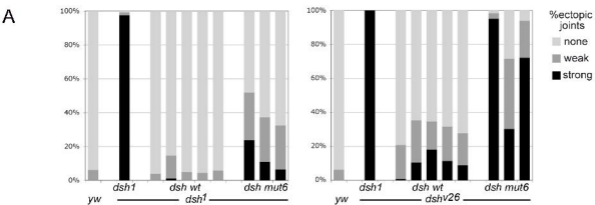
**C**

DVEZ IIX `ETDGDYKSWLQREYGRK`  
 DVEZ IIX `V...PD...GV...QRFQYK`  
 DVE DQ `V...RD...LV...KQKQYR`  
 NIS DQ `F...RQ...DG...FRGQYR`  
 AKS1 IIX `V...GQ...EL...TQKQYR`

**D**

mut1: D39S + V43E  
 mut2: D39S + V43Q  
 mut3: D39S  
 mut4: V43E  
 mut5: V43Q  
 mut6: K46V + Q47A





wild type

*dsh1*

