REGULATION OF PLANAR CELL POLARITY BY THE DACHSOUS-FAT PATHWAY

by

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ABSTRACT OF THE DISSERTATION

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Planar cell polarity is the polarization of cells within the plane of a tissue. The Dachsous-Fat pathway plays a key role in the regulation of planar cell polarity and growth during the development of an organism. The transmembrane cadherins Fat and Dachsous from neighboring cells interact heterophilically and regulate the localization of the unconventional myosin Dachs in a cell. Four-jointed, a Golgi-localized kinase modulates the binding between Dachsous and Fat. Dachsous and Four-jointed are expressed in tissue-wide gradients, which influence Fat activity and direct the polarized membrane localization of Dachs.

We demonstrate that differential expression of Dachsous or Four-jointed can modulate Dachs polarization at a distance in wing discs. This indicates that Dachsous and Four-jointed gradients can be measured over long range in a tissue through propagation
of polarity. We also show that Dachsous and Fat are partially polarized along the endogenous Dachsous and Four-jointed gradients, providing a mechanism for propagation of polarity. Through directed membrane targeting of Dachs, we show that membrane localization of Dachs influences both planar cell polarity and the Hippo signaling pathway. These studies help in understanding the mechanisms involved in establishment and maintenance of planar cell polarity.

The Frizzled pathway is another key pathway that regulates planar cell polarity, but its relationship with the Dachsous-Fat pathway was unclear. We demonstrate that Dachs and Dachsous can independently interact with a Frizzled pathway component, Spiny-legs, and direct its localization in vivo. Thus, the Dachsous-Fat pathway provides directional input to the Frizzled pathway by influencing the localization of Spiny-legs. These studies help in understanding how planar cell polarity is regulated in various tissues through coordination between Dachsous-Fat and Frizzled pathways. These studies also reveal that Spiny-legs and its isoform Prickle can respond to distinct planar cell polarity signals and allow the cells to compare and choose between these competing signals to direct polarity robustly in one direction.

Thus, our results identify a mechanism for propagation of planar cell polarity through the tissue, establish the significance of Dachs membrane localization in Dachsous-Fat signaling and identify a molecular mechanism for crosstalk between the two planar cell polarity pathways.
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Dedication

This thesis is dedicated to my parents.
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CHAPTER 1

General Introduction
Planar cell polarity

Planar cell polarity (PCP) refers to the polarization of cells within the plane of the tissue and orthogonal to the apical-basal axis. This allows the cells to obtain directional information with respect to the body axes. PCP is essential during the development of multicellular animals to form organs with specific morphology and organization of cellular structures.

PCP was initially described in insects and has been found to have similar functions in a wide range of animals, including mammals. PCP is involved in the formation of patterns such as oriented outgrowths of hairs on insect wing and mammalian skin, polarized beating of cilia, orientation of ommatidia in the compound eye of insects and stereocilia in the inner ear of mammals. PCP can influence the orientation of mitotic spindle during cell division, which is important in determining the shape of the organ. PCP is also essential for coordinated and directed cell movements during various processes like convergent extension in vertebrate embryos, collective migration of neural crest cells, neuronal migration and elongation of developing cartilages. Oriented cell division and coordinated cell movements are also necessary for branching morphogenesis, such as in uterine bud branching during kidney development and lung morphogenesis. PCP signaling can also influence the direction of growth cones during axon guidance. Thus, PCP signaling controls a wide array of developmental and physiological processes in mammals, highlighting its importance. Dysregulation of PCP signaling can cause severe abnormalities such as defects in neural tube closure, cystic kidney disease, loss of hearing due to ciliary disease and left/right patterning defects.
There are two molecular systems that are involved in establishment and maintenance of PCP: Dachsous-Fat (Ds-Fat) PCP pathway and Frizzled (Fz) PCP pathway. The components of these pathways localize asymmetrically in the apical or sub-apical region of the cell. These PCP pathway proteins signal to the downstream polarity effectors to convert the directional information into polarized output. The PCP pathway proteins also interact between neighboring cells to coordinate the polarity across the tissue. The upstream tissue-level polarity cues are provided in form of morphogen gradients, which regulate the expression levels and localization of PCP pathway proteins. This establishes an initial bias in cellular polarity, which further amplifies and spreads through the tissue. However, the complete list of global polarity cues still remains unknown. It is also not completely clear how the components of the two PCP pathways interact to align their asymmetries along the tissue axes. Moreover, there are some gaps in the understanding of how the PCP pathways signal to the downstream PCP effectors. The research in this thesis attempts to address some of these questions in the field of PCP.

**Ds-Fat PCP pathway**

The Ds-Fat PCP pathway is crucial in regulating growth and tissue polarity. The components of Ds-Fat pathway include the transmembrane cadherins Fat and Dachsous (Ds), the unconventional myosin Dachs and a Golgi-localized kinase Four-jointed (Fj). Fat and Ds from neighboring cells interact heterophilically and regulate the localization of Dachs [1-4] (Fig. 2). Upon interaction with Ds, activated-Fat inhibits the accumulation
of Dachs on the membrane [1]. Four-jointed phosphorylates cadherin domains of Fat and Ds to modulate the binding between them [5-7]. Fat is a 5147-amino acid protein containing 34 cadherin domains, 5 epidermal growth factor (EGF)-like domains and 2 laminin-G like domains in its extracellular region [3]. The cytoplasmic domain of Fat can be phosphorylated by Discs overgrown (Dco), interacts with Lowfat (Lft) and Atrophin, and contain distinct domains for regulating PCP and Hippo signaling [8-13]. Ds is a 3503-amino acid protein containing 27 cadherin domains in its extracellular region [2]. Dachs is a 1232-amino acid Myosin family protein containing ATP-binding domain, actin binding domain and active thiol region [1]. In myosin proteins, the active thiol region is involved in ATPase activity and actin binding [14, 15]. However, it has been reported that Dachs does not have ATPase activity and is able to bind to actin in an ATP-independent manner, suggesting that Dachs may function as a scaffold protein [16]. Localization of Dachs is also regulated by DHHC palmitoyltransferase Approximated (App) and F-box protein Fbxl7 [17-19].

Ds and Fj are expressed in opposing gradients in various *Drosophila* organs, including wing and eye imaginal discs and abdominal segments [2, 20-23] (Fig. 2). Graded expression of Ds and Fj results in polarization of Dachs along the direction of these gradients [24-26]. However, it was not clear how cells sense the Ds-Fj gradients and translate the information into polarized localization of Dachs. We demonstrate that PCP can propagate over a long range in the tissue through coupling of polarity between adjacent cells (Chapter 2). This provides a mechanism for long range sensing of gradients by the cells. Also, we demonstrate that Ds and Fat get asymmetrically localized along the direction of gradients, allowing for coupling of polarity between adjacent cells and
polarization of Dachs along the direction of these gradients. The amount of Dachs on the membrane correlates with the activation of Hippo signaling by the Ds-Fat pathway, while the direction of Dachs polarization correlates with PCP [27, 28]. Thus, regulation of Dachs localization is crucial for Ds-Fat signaling. However, the mechanism by which Fat affects Dachs localization, and the importance of Dachs localization on membrane was not clear. We demonstrate that Fat can influence the dynamics of Dachs on membrane, and Dachs membrane localization is important for regulation of both PCP and Hippo signaling by the Ds-Fat pathway (Chapter 3).

Dysregulation of Ds-Fat pathway affects both tissue polarity and growth. Mutation in fat results in overgrowth while mutation in dachs results in reduced growth. Mutations in fat, ds and fj result in disturbed hair and bristle polarity in the wing and the abdomen, and also affect ommatidial polarity in the eye [22, 29, 30]. Although mutation in dachs results in mild polarity phenotypes, it can suppress the polarity phenotype of fat and ds mutants in the wing and the abdomen, indicating its role in PCP regulation [1]. The Ds-Fat pathway also affects the orientation of cell division [31, 32], which is important in determining the shape of the organs [33]. Mutants of dachs, ds or fat have shortened P-D axis relative to the A-P axis, resulting into rounder wings. Fat4 and Dchs1, the mammalian homologs of Fat and Ds can influence PCP in various organs. Dchs1 and Fat4 are expressed in opposing gradients in the mouse hindbrain and influence the migration of facial branchiomotor neurons [34]. Mutations in Fat4 or Dchs1 cause alterations in hair cell orientation in the cochlea, short cochlea, cystic kidneys, altered neuronal migration and defects in oriented cell division [35, 36]. Recently, it has been reported that mutations in DCHS1 causes mitral valve prolapse in humans [37].
**Hippo signaling pathway**

The Hippo pathway is a major growth regulatory pathway that is important in control of organ size. The Hippo kinase cassette at the core of the pathway consists of Ser/Thr kinases Hippo (Hpo) and Warts (Wts), scaffolding protein Salvador (Sav) that interacts with Hpo and Wts, and Mob-as-tumor-suppressor (Mats) which acts as co-activator for Wts [38-41]. The Hpo-Sav kinase complex phosphorylates the Wts-Mats kinase complex to activate it. Multiple upstream inputs regulate the activation of Hippo kinase cassette, which in turn regulates the nuclear localization of transcriptional co-activator Yorkie (Yki) through phosphorylation at Ser168 [42]. Once inside the nucleus, Yki interacts with various DNA binding partners such as Scalloped (Sd), Homothorax (Hth) and Mad to regulate the transcription of distinct sets of target genes. These target genes include *diap1*, *bantam*, *cyclinE* and *myc*, which promote growth and cell survival. Thus, the kinase cassette or upstream regulators act as tumor suppressor genes and loss-of-function mutations in these genes result in overgrowth, whereas Yki acts as a proto-oncogene and loss-of-function mutations in Yki results in reduced growth.

Various upstream regulators of Hippo signaling include Kibra, Merlin (Mer), Expanded (Ex), Discs large (Dlg), Lethal giant larvae (Lgl), Scribble (Scrib), Crumbs (Crb) and Ajuba (Jub). New regulators that impinge on Hippo pathway to activate the kinase cassette are continuously being identified. The Ds-Fat pathway also acts as one of the upstream regulator of the Hippo pathway to regulate growth [43]. Fat can influence the levels of Wts and Ex to regulate Yki activity, and Dachs is required for both these effects. Dachs binds to the LIM domain protein Zyxin (Zyx) to stimulate a conformational change in Zyx, exposing its LIM domains. This enables the binding of
Zyx to Wts, thus regulating the levels of Wts [44]. The Ds-Fat pathway can also influence Yki activity through phosphorylation-dependent inhibition of Wts by the WD40 repeat protein Riquiqui (Riq) and the DYRK-family kinase Minibrain (Mnb), which function downstream of Ds [45].

**Fz PCP pathway**

In the Frizzled PCP pathway, the proteins are segregated into two complexes present on the opposite sides within a cell (Fig. 2). In wing imaginal discs, the distal complex consists of the membrane spanning proteins Frizzled (Fz) [46] and Flamingo (Fmi) (also known as Stan) [47], and the cytoplasmic proteins Dishevelled (Dsh) and Diego (Dgo) [48-51]. The proximal complex consists of the membrane spanning proteins Strabismus (Stbm) (also known as Vang) and Flamingo (Fmi), and the cytoplasmic protein Prickle (Pk-Sple) [52, 53]. The proximal and distal complexes interact between neighboring cells to regulate PCP [54, 55]. Prickle is a LIM domain protein and is involved in the intracellular feedback loop to maintain asymmetry of the distal and proximal complexes by inhibiting accumulation of Dsh and Fz on proximal side of the cell [56]. Prickle is present in two isoforms: Prickle (Pk) and Spiny-legs (Sple) that differ at their N-terminus [57]. These proteins mediate their PCP signaling function through their PET and LIM domains [58]. These isoforms are genetically important in regulating PCP in different tissues, presumably due to the difference in their endogenous expression levels. Pk is expressed at higher levels than Sple in wing disc during larval development [59] whereas in eye disc, Sple is expressed at higher levels than Pk [60]. Loss of *pk*
affects PCP strongly in wing, notum and posterior compartment of the abdominal segment, whereas loss of sple affects PCP strongly in leg, eye and anterior compartment of the abdominal segment. However, loss of both the isoforms has mild effect on PCP in the whole body. Hence, it was earlier proposed that the balance between the Pk and Sple isoforms is important for normal PCP [57]. However, we now demonstrate that rather than balance, Pk and Sple would provide competing polarizing cues and cells would choose between these cues based on the protein abundance (Chapter 4).

**Interconnection of the Ds-Fat and Fz pathway**

The relationship between the Ds-Fat and Fz PCP pathway has not been clear. In wing and eye, Ds and Fj gradients are aligned along the proximal-distal axis or pole-equator axis respectively (Fig. 3). It has been shown that in wing, the Fz pathway components are polarized in ds, fj or fat mutants, but the direction of orientation is no longer along the tissue axes [61]. It has also been shown that Fz PCP signaling can occur in two phases during pupal development. The early phase of this signal depends on Sple isoform and its direction is controlled by the gradients of Ds-Fat pathway [62]. Hence, it was proposed that the Ds-Fat pathway would provide global polarity information in form of Ds/Fj gradients, and act upstream of Fz PCP pathway. This would align the direction of polarization with tissue axes. However, the direction of Ds and Fj gradients is opposite in the anterior vs. posterior compartment of the abdominal segments (Fig. 3), but the hair always point in the posterior direction [22]. Moreover, mutant or overexpression clones of ds, fj or fat in abdomen can affect PCP non-autonomously in absence of Fz pathway.
components, suggesting that the Ds-Fat pathway can polarize cells independently of the Fz pathway [63]. Based on these observations, it has been proposed that the two PCP pathways would act in parallel to regulate PCP in abdomen. This is supported by the observation that in the larval abdominal denticle belts, loss of both Ds-Fat and Fz pathway proteins have severe effect on denticle PCP compared to loss of proteins from single pathway [63, 64].

Several studies have tried to establish the molecular link between Ds-Fat and Fz PCP pathway. It has been reported that vesicles containing Fz and Dsh are transported along apical non-centrosomal microtubules (MTs) towards distal side of a cell, and this proximal-distal alignment of MTs depends on Ds-Fat pathway [65-67]. The polarity of these MTs is controlled by the Pk and Sple isoforms, such that the plus ends of MTs are biased towards either high end or low end of Fj gradient, depending on whether Pk or Sple is the predominant isoform respectively [59]. It has also been reported that Dachs can directly interact with Sple and Pk, and influence its localization in wing disc [60]. We additionally demonstrate that Dachs and Ds can independently interact with N-terminus of Sple, and influence its localization in wing and eye imaginal discs and abdomen (Chapter 4). These observations suggest that the Ds-Fat pathway can provide directional input to the Fz pathway by influencing the localization of Sple.

**Model organs to study PCP**

*Drosophila* has been an important model system for studies on PCP. The PCP pathways described above were first discovered in *Drosophila* and are conserved in other
organisms including mammals. The *Drosophila* tissues used for studying PCP include wing, leg, eye, notum and abdomen. PCP pathways regulate the orientation of epithelial structures such as cuticle hairs and sensory bristles towards distal side (wing and legs) and towards posterior side (notum and abdomen) (Fig. 1A, A’, A” and C). In case of eye, PCP pathways regulate the chiral arrangement of photoreceptor cells in the ommatidia (Fig. 1B). PCP is also manifested in form of cuticular ridges on the wing and denticles on larval cuticle [64, 68]. Apart from epithelial structures, PCP pathways also influence the orientation of cell division and cell rearrangements, which plays an important role in elongation of wing during growth [32]. The main focus of the experiments to investigate PCP regulation described in this thesis is using wing, eye and abdomen.

**Wing**

The wing imaginal disc is subdivided into anterior and posterior compartments by the A-P boundary, and into dorsal and ventral compartments by the D-V boundary (Fig. 2A). The morphogens Decapentaplegic (Dpp) and Wingless (Wg) are expressed along the A-P boundary and D-V boundary respectively. These morphogens form a gradient in the wing imaginal disc, which regulates the expression of downstream genes including the Ds-Fat pathway genes Ds and Four-jointed (Fj). Hence, Ds expression is graded from periphery (proximal region) of wing pouch to center (distal region) (Fig. 2A’) and Fj expression is graded from center of wing pouch to periphery (Fig. 2A”). During pupal
development, the wing imaginal disc evaginates and the epithelial tissue elongates along the proximal-distal axis to form the adult wing.

Eye

In eye imaginal disc, the morphogen Wg is expressed at the poles and diffuses towards equator, aligning the gradients along the direction of pole to equator [69]. Ds expression is graded from pole to equator (Fig. 2B’), whereas Fj expression is graded from equator to pole (Fig. 2B”). The cells in eye imaginal disc are initially undifferentiated and unpatterned. They differentiate into photoreceptor cells at the morphogenetic furrow, which initiates at the posterior region of the eye disc and proceeds towards anterior side. Each ommatidium consists of 8 photoreceptor cells and the orientation depends upon the specification of R3 versus R4 photoreceptor cell, which in turn depends on Notch signaling [70, 71] (Fig. 2B).

Abdomen

The abdominal segment is subdivided into anterior and posterior compartments. The morphogen Hh is expressed in the posterior compartment and diffuses into the anterior compartment [72]. The gradients of Ds and Fj run in opposite direction in the anterior and posterior compartments [22] (Fig. 2C, C’ and C”). However, the hairs and the bristles in the adult cuticle always point posteriorly.
FIGURES

Figure 1. Manifestations of PCP in various tissues of *Drosophila*

(A) Schematic of wild type adult wing to show panels for wing margin bristles (A’) and wing blade hair (A”). Proximal is towards the left of the image and distal towards the right. Anterior is towards the top of the image and posterior towards bottom.

(B) Wild type adult eye showing the arrangement of R1-R7 photorecepter cells in the ommatidia. The horizontal dotted line represents the equator. (figure adapted from [73]).

(C) Hair and bristle polarity in wild type adult abdomen. Anterior is towards the top of the image and posterior towards bottom.
Figure 2. Gradients of Ds and Fj in various tissues of *Drosophila*

(A) Cartoon illustrating the A-P and D-V boundaries in wing imaginal disc.

(A’ and A”) Ds and Fj expression gradients in wing imaginal disc, revealed by *ds-lacZ* enhancer trap (A’) and *fj-lacZ* enhancer trap (A”). (figure adapted from [27])

(B) Cartoon illustrating the arrangement of photoreceptor cell cluster in eye imaginal disc.

(B’ and B”) Ds and Fj expression gradients in eye imaginal disc, revealed by *ds-lacZ* enhancer trap (B’) and *fj-lacZ* enhancer trap (B”). (figure adapted from [28])

(C) Graph illustrating the slope of Ds and Fj gradients in the anterior and posterior compartment of abdominal segments.

(C’ and C”) Ds and Fj expression gradients in ventral abdomen, revealed by *ds-lacZ* enhancer trap (C’) and *fj-lacZ* enhancer trap (C”). (figure adapted from [22])
Figure 3. The Ds-Fat and Fz PCP pathway

(A and B) Schematic illustrating the subcellular localization of Ds-Fat (A) and Fz PCP pathway (B) components in cells of wing disc.
A) Ds-Fat PCP pathway

B) Fz PCP pathway
CHAPTER 2

Propagation of Dachsous-Fat planar cell polarity

Abhijit Ambegaonkar, Guohui Pan, Madhav Mani, Yongqiang Feng and Kenneth D. Irvine

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Author contributions:
Abhijit Ambegaonkar, Guohui Pan, Yongqiang Feng and Kenneth D. Irvine conceived and designed the experiments. Abhijit Ambegaonkar conducted the experiments. Guohui Pan and Yongqiang Feng constructed the Drosophila stocks used in analysis of Ds and Fat polarization. Madhav Mani developed the image analysis method for quantifying Dachs polarity.
SUMMARY

The Fat pathway controls both planar cell polarity (PCP) and organ growth [28, 74]. Fat signaling is regulated by the graded expression of the Fat ligand Dachsous (Ds) and the cadherin-domain kinase Four-jointed (Fj). The vectors of these gradients influence PCP [74], whereas their slope can influence growth [27, 75]. The Fj and Ds gradients direct the polarized membrane localization of the myosin Dachs, which is a crucial downstream component of Fat signaling [1, 32, 43]. Here we show that repolarization of Dachs by differential expression of Fj or Ds can propagate through the wing disc, which indicates that Fj and Ds gradients can be measured over long range. Through characterization of tagged genomic constructs, we show that Ds and Fat are themselves partially polarized along the endogenous Fj and Ds gradients, providing a mechanism for propagation of PCP within the Fat pathway. We also identify a biochemical mechanism that might contribute to this polarization by showing that Ds is subject to endoproteolytic cleavage and that the relative levels of Ds isoforms are modulated by Fat.
RESULTS and DISCUSSION

Propagation of Fat-PCP through the Wing Disc

Two complementary mechanisms for coordination of PCP have been suggested [76]. One relies upon local interactions that enable the polarity of one cell to be coupled to that of its neighbors. The other relies upon organ-wide gradients, whose vectors could be interpreted by each cell. Two PCP pathways have been identified in metazoans, a Frizzled-dependent pathway (Fz-PCP) and a Dachsous (Ds)- and Fat-dependent pathway (Fat-PCP) [76]. Fat-PCP is regulated by Ds and Fj gradients, but it has remained unclear whether coupling mechanisms that coordinate polarity between adjacent cells also contribute to Fat-PCP.

Dachs is polarized along the Fj and Ds gradients (Figures 1B–1E) [1, 27]. Dachs localization can be altered by manipulating Fj or Ds expression, but initial experiments only examined Dachs repolarization within cells with altered Fj or Ds [1, 32]. We reasoned that if the polarity of neighboring cells is coupled, then the influence of Fj or Ds could propagate through a tissue. This was examined using a tagged dachs transgene (AyDachs:Cit) that could be expressed in clones independently of manipulations of Fj or Ds. AyDachs:Cit was crossed into flies in which Ds or Fj expression were either increased (using UAS transgenes) or decreased (using UAS-RNAi transgenes) within posterior cells under hedgehog (hh)-Gal4 control.

Within the medial wing (near the hh expression border), Dachs is preferentially detected along the sides of cells closest to the dorsal-ventral (DV) boundary, which can be identified by expression of Wingless (Wg) (Figures 1B and 1C). When Ds was
overexpressed in posterior cells, Dachs was relocalized in nearby anterior cells, defining a reoriented vector of polarization (Figure 2A). This repolarization was detected not only in adjacent cells but also a few cells away, reflecting a propagation of altered PCP from the Ds expression boundary into the anterior compartment (Figure 2A).

Repolarization of Dachs within anterior cells was also detected when Fj was overexpressed in posterior cells (Figure 2B) or when Ds or Fj were depleted from posterior cells, and this repolarization could be detected at a distance. When Fj was depleted or Ds was overexpressed, Dachs was relocalized to the sides of cells farthest from the anterior-posterior (AP) boundary (Figure 2A, 5B). Conversely, when Fj was overexpressed or Ds was depleted, Dachs was relocalized to the sides of cells closest to the AP boundary (Figure 2B, 5A). This parallels wild-type (WT), because Dachs normally accumulates on the sides of cells closest to where Fj is highest and Ds is lowest.

To quantify the distance over which Dachs could be repolarized, we scored AyDachs:Cit clones in UAS-ds, UAS-fj, UAS-RNAi-ds, or UAS-RNAi-fj expressing wing discs. These clones were subdivided into distal wing, proximal wing, or dorsal hinge. Within each disc, the number of cells from the AP boundary to the farthest cell with evident Dachs repolarization was recorded, as was the distance to the closest cell in which Dachs polarity appeared normal. This revealed that the range of repolarization varied from zero to six cells, depending upon the genotype and clone location (Figures 2C–2G).

For UAS-ds and UAS-RNAi-fj, the range of repolarization was longest within the distal wing and shortest within the hinge (Figures 2C–2G). Conversely, for UAS-fj and
*UAS-RNAi-ds*, the repolarization range was longest within the hinge and shortest within the distal wing. Because Ds expression is normally highest proximally and lowest distally, whereas Fj is highest distally and lowest proximally (Figures 1D and 1E), these results imply that the range of repolarization depends upon the difference in expression. This suggests that there is a gradual dissipation of repolarization: a larger expression difference would generate a stronger repolarization of neighboring cells, which propagates farther than an initially weaker repolarization. In addition, longer extents of repolarization were observed in proximal regions, particularly within the hinge, as compared to distal regions. For example, both expression of *UAS-ds* in the distal wing and *UAS-RNAi-ds* in the hinge result in strong differences in Ds expression, but the range of repolarization was longer in the hinge (Figure 2C).

We confirmed these effects of Ds and Fj on Dachs polarization through an independent approach involving quantitative image analysis. By taking advantage of a slight offset between the membrane localization of Dachs versus E-cadherin, we were able to computationally derive a mean vector of Dachs polarization within individual cells. This was used to score anterior Dachs polarization in discs with altered Ds or Fj expression in posterior cells. This analysis confirmed that Dachs could be repolarized at a distance, with differences in the extent of repolarization between distal and proximal cells (Figures 5D–G).

The observation that Fat-PCP can propagate through tissues establishes that differences in Fj or Ds expression can be sensed by cells at a distance through coupling of polarization between neighboring cells. This implies that gradients can be measured across a tissue, rather than just between neighboring cells.
**Polarization of Ds and Fat**

One potential mechanism for propagation of Fat-PCP is polarization of Fat and Ds. Overexpression or mutation of Fat or Ds can relocalize its binding partner (i.e., Ds or Fat, respectively) [1, 4, 30, 61, 77]. Similarly, mutation or overexpression of Fj can alter localization of Ds and Fat [1, 7, 30, 61, 77]. However, these manipulations cause strong changes in relative expression, and it had remained uncertain whether Ds and Fat are normally polarized within endogenous gradients. We could not discern polarization by direct examination of protein staining. Detecting polarization by making clones of cells expressing a tagged protein, as described above for Dachs, would obscure any normal polarization of Ds and Fat, because of the relocalization of Fat or Ds that occurs when their binding partner is overexpressed. Thus, we employed a more complex approach involving Bac clones expressing tagged forms of genomic transgenes [78].

A 39 kb genomic fat construct with a V5-tag at the Fat N terminus has been described [10]. We generated an equivalent untagged genomic fat construct and both untagged and hemagglutinin (HA)-tagged Ds genomic constructs, comprising 109 kb surrounding ds (Figures 6A–D). These constructs were inserted at the same location, using phiC31-mediated recombination [79]. They encode functional proteins, because they can rescue their respective mutants. We constructed flies heterozygous for the untagged and tagged versions; they contain four copies of fat or ds (two from the WT allele and two from the transgenes), but this does not result in any visible phenotypes. Induction of recombination between the tagged and untagged transgenes results in clones
of cells that either have two tagged transgenes and no untagged transgenes, or two untagged transgenes and no tagged transgenes (Figure 6E), with the total copy number of Fat or Ds unaltered. We then examined the staining of tagged proteins along the edges of clones of cells with only untagged Fat or Ds (Figures 3A, F; 7E–G). We also stained for the endogenous proteins to ensure that differences detected reflected polarization, as opposed to differences in focal planes or expression levels and considered the intensity of staining of a clone marker (GFP), so that comparisons would be made between cells with equal numbers of tagged transgenes.

In preliminary analysis, Ds protein appeared polarized, with stronger HA staining detected on cells proximal to unlabeled clones than on cells distal to unlabeled clones (Figures 3A and 3B; Figure 7F). Because this staining comes from neighboring cells (Figure 6E), it is indicative of a polarized, distal membrane localization for Ds. Conversely, Fat did not appear polarized. To confirm this, we collected images of 80–100 clones from both the tagged-Fat and tagged-Ds experiments, assigned random numbers, and then scored blind (i.e., without knowledge of whether Ds or Fat was being scored). In this blind scoring, 74% of Ds clones were scored as revealing a polarization of Ds localization toward the distal side of cells, 8% were scored as revealing a proximal polarization, and 18% were scored as revealing a lack of polarization (Figure 3C). By contrast, 7% of Fat clones were scored as revealing a polarization of Fat localization toward the distal side of cells, 43% were scored as revealing a proximal polarization, and 50% were scored as revealing a lack of polarization (Figure 3E). Polarization of Ds or Fat could also sometimes be identified when a single cell expressing a tagged transgene was
bordered on both proximal and distal sides by cells lacking tagged transgenes (Figures 3A, F).

Thus, Ds is polarized toward the distal sides of cells. However, in contrast to Dachs, for which membrane staining is normally detected on only one side of a cell, membrane staining of Ds is often detected on both sides of a cell but at unequal levels. Fat also appears to be polarized, although its polarization is weaker, such that it often falls below our ability to discern it. One possible explanation for this is that Ds expression is relatively low within the wing pouch (Figure 1D), whereas Fat expression is relatively high [8]; a polarized localization that is coupled to Ds-Fat binding would thus affect a greater fraction of the available Ds than of the available Fat. The more robust polarization of Dachs compared to Fat and Ds suggests that there is an amplification mechanism that operates downstream of Fat and Ds localization to enhance Dachs polarization.

The polarization of Ds and Fat identifies modulation of their localization as a potential mechanism for propagation of Fat-PCP. Moreover, detection of this polarization in WT implies that it normally makes a contribution to PCP. Because proximal and distal cells are characterized by differences in the levels of Ds and Fat, the hypothesis that polarization of Ds and Fat contributes to propagation of PCP suggests explanations for the greater range of Dachs repolarization in the hinge compared to the distal wing. For example, if propagation of Fat-PCP depends upon Ds, then repolarization could be more extensive where Ds expression is higher than where it is lower.
The distal localization of Ds in wing cells parallels the distal localization of Dachs. Moreover, both Dachs and Ds often appear to have a punctate localization profile at the membrane, rather than smooth continuous staining [27]. Their correlated localization extends to these puncta (Figure 3G). However, Ds is not required for Dachs membrane localization, because strong, unpolarized membrane localization of Dachs is detected in ds mutant discs (Figure 7B), just as it is in fat mutants [1].

**Processing of Ds and Its Modulation by Fat**

In Fz-PCP signaling, maintenance and propagation of polarity depend both upon intercellular binding between distinct of membrane complexes and an intracellular, mutual antagonism between these complexes [80]. Intercellular binding between Fat and Ds has been described [4, 7, 30, 61, 77]. To investigate the possibility of intracellular antagonism between Fat and Ds, we asked whether there are posttranslational modifications of one protein that depend upon the other. An influence of Ds on Fat phosphorylation has been described, but it only influences Fat-Hippo signaling, not Fat-PCP [10, 81].

Examination of Ds protein in wing disc lysates revealed that in addition to a band near the top of the gel, which could represent full length Ds (Ds-FL), two smaller bands, with mobilities corresponding to approximately 150 kd (Ds-C150) and 210 kd (Ds-C210), were detected (Figure 4A). These bands were confirmed as Ds polypeptides by their absence from lysates of ds mutant discs (the antisera also recognizes nonspecific bands (NS), which are present in ds mutants). These bands represent C-terminal
fragments, because the Ds antisera is directed against the cytoplasmic domain [21]. There are several potential mechanisms by which smaller Ds polypeptides could be generated, such as proteolytic cleavage, alternative transcription starts, or alternative splicing. A prediction of the endoproteolytic cleavage hypothesis is the existence of complementary N-terminal Ds polypeptides. This was tested using our genomic HA-tagged Ds transgene. In these animals, anti-HA recognized a band near the top of the gel with the same mobility as the largest band detected by anti-Ds (Ds-FL) and also two smaller polypeptides, with mobilities of approximately 270 kd (Ds-N270) and 220 kd (Ds-N220) (Figure 4B). These observations suggest that Ds can be endoproteolytically processed at one of two alternative sites, a more N-terminal site, leading to Ds-N220 and Ds-C210 polypeptides, and a more C-terminal site, leading to Ds-N270 and Ds-C150 polypeptides (Figure 4C). In most of the wing disc, the anti-Ds and anti-HA localization profiles were not distinguishable (Figures 7D–F). The overall similarity between HA and Ds staining patterns suggests that the N- and C-terminal halves of Ds remain associated.

The total amount of Ds was not affected by fat mutation (Figure 7C). However, absence of fat had a significant effect on Ds isoforms, as Ds-C210 increased, whereas Ds-C150 decreased (Figures 4A and 4D). The observation that these are affected in opposite ways is consistent with the hypothesis that Ds is subject to alternative, mutually exclusive processing pathways. More importantly, it suggests that Fat modulates this processing. When the N-terminal half of Ds was examined, Ds-N270 was decreased in fat mutants, consistent with a stoichiometric relationship between Ds-N270 and Ds-C150 (Figures 4B–D). A subtle increase in Ds-N220 was observed, but it was not statistically significant (Figure 4D). This lack of effect on Ds-N220 does not fit the simple model of alternative...
processing, and there may be additional effects of fat on Ds-N220. Although further studies will be needed to clarify the mechanism by which these Ds polypeptides are generated, and their significance to Fat signaling, the observation that Fat influences the distribution of Ds isoforms is significant in that it identifies a posttranslational influence of Fat on Ds that might contribute to Ds polarization.

**Establishment of Polarity by Ds and Fj Gradients**

Our observation that differences in Fj or Ds expression can alter Fat PCP at a distance and that Ds, and to a lesser extent Fat, is polarized within the wing, together with other recent studies [25, 26], imply that establishment of polarity in the Fat PCP system relies not just upon direct interpretation of Fj and Ds gradients but also upon amplification and propagation of PCP. To achieve this, PCP models incorporate both asymmetric intercellular signaling and antagonistic intracellular interactions between complexes that localize to distinct sides [80, 82]. Intercellular binding between Ds and Fat is well established, but on its own, this would not propagate polarity from cell to cell. However, incorporation of a local, intracellular antagonism of Ds by Fat activity could polarize Ds localization, which could then enable Fat-PCP to propagate. We hypothesize that Fat regulates Ds by influencing production or stability of processed Ds isoforms.

The propagation of polarity means that Fat-PCP is influenced not only by the local gradient but also by differential expression at a distance. Strong repolarization of Dachs was dependent upon having substantial differences in expression. Notably, strong differences in expression of both Fj and Ds normally occur in the proximal wing (Figures
1D, E), and these differences have significant effects on Fat activity [77, 83]. Both our measures of the range of Dachs repolarization and mathematical modeling [84] suggest that the Fj/Ds expression boundary in the proximal wing would not be sufficient to direct Fat-PCP across 30 or more cells, as would be required at late third instar. However, at early third instar, when the developing wing is small, a mechanism that propagates PCP from an expression boundary for several cells could in principle be sufficient to establish PCP throughout the wing. Once established, the mechanisms that allow Fat-PCP to propagate could also help maintain Fat-PCP as the wing grows. In this case, the Fj and Ds boundaries at the edge of the developing wing would be the main drivers of polarity, rather than the shallow gradients of their expression within the wing itself.
EXPERIMENTAL PROCEDURES

*Drosophila Stocks and Crosses*

Fly stocks used for investigation of Ds and Fat polarization included:

\[ yw \text{ hs-FLP[122]}; If/CyO; Ubi-GFP P[acman-HA:ds}^+{-attP68A}] \text{ FRT80/TM6b} \]

\[ yw \text{ hs-FLP[122]}; If/CyO; P[acman- ds}^+{-attP68A}] \text{ FRT80 /TM6b} \]

\[ yw \text{ hs-FLP[122]}; If/CyO; Ubi-GFP P[acman-V5:fat}^+{-attP68A}] \text{ FRT80/TM6b} \]

\[ yw \text{ hs-FLP[122]}; If/CyO; P[acman- fat}^+{-attP68A}] \text{ FRT80 /TM6b} \]

Twin spots were induced in heterozygous larvae by heat shock (38°C for 1h) during the second instar and early third instar, and larvae were fixed at mid-third instar.

Fly stocks used for investigation of Dachs repolarization included:

\[ yw; act>CD2,y+>Dachs:Cit (AyDachs:Cit)/CyO-GFP; hh-Gal4 UAS-dcr2/TM6b \]

\[ yw \text{ hs-FLP[122]}; If/CyO; UAS-RNAi-ds[vdrc4313]/TM6b \]

\[ yw \text{ hs-FLP[122]}; If/CyO; UAS-RNAi-fj[vdrc6774]/TM6b \]

\[ yw \text{ hs-FLP[122]}; UAS-ds[III]/TM6b \]

\[ yw \text{ hs-FLP[122]}; UAS-fj[146.3]/TM6b \]

Flip-out clones of Dachs:Cit were induced by heat shock (36°C for 40 min) during early third instar, and larvae were fixed at mid-third instar.
**Plasmid Constructs and Primers**

The plasmid pUAST-Dachs:Cit (gift of Binnaz Staley) was constructed by inserting a coding sequence for a short linker (5'-ctggcggcggcggc-3') followed by the coding sequence for Citrine at the C-terminus of the Dachs sequence in pUAST-Dachs:V5 [1]. Restriction sites AvrII and KpnI were introduced into the 5’ end and BglII into the 3’ end of the Dachs:Cit coding sequence using primers dcit_AK_fp (5’-CTCGAACCACGCCTAGGACGGGTACCCTCTGAATAGGGAATTGGGAATTC-3’) and dcit_B_rp (5’-CGTGGTT CGAAAGATCTCGATTACTTGTA CAGCTCGTCCATG-3’) respectively. The FRT cassette in the vector pWAYGal4Nhe [85] was released with NheI and BamH1 and Dachs:Cit was inserted. The FRT cassette encoding y+ and CD2 was then inserted into the KpnI site between the Actin5C promoter and Dachs:Cit to construct the plasmid pWAYC Dachs:Cit. This plasmid was used for injection to generate transgenic flies expressing Flip-out clones of Dachs:Cit (AyDachs:Cit).

The attB-P[acman- ds+] Bac clone was constructed by sequentially recombining portions of two Bac clones (BACPAC Resources Center) into attB-P[acman]-ApR using protocols described by [78]. In brief, BACR48E8 was first recombined into a donor plasmid through homolog arms LA1 (created by PCR using primers LA1-NotI-F (5’-AATA GCGGCCGC GATGGCTATTTAGCATAGA-3’) and LA1-BamHI-R (5’-GC GGATCC ACAGAATACTCGTCC TTTTCA-3’)), and RA1 (created by PCR using primers, RA1-BamHI-F (5’-T GGATCC AGGCAGAAGACTCA AAAGATG-3’) and
RA1-PacI-R1 (5’-CCC TTAATTAA TGTATTGGTGATGAATAAGG-3’), forming homolog arm RA2. Then BACR30G21 was recombined between LA2 (created by PCR using primers LA2-AcsI-F (5’-A GCGCGCCC TTTTCTCAGATAACACC-3’) and LA2-NotI-R (5’-AATA GCGGCCGC TAATCCCGATTGGGCTCACT-3’)), and RA2. The whole genomic region incorporated is 109082bp, starting from 58bp 3’ of Eaat2, including Hsp60B, dachsous and the first 77bp of CG2839. The whole plasmid is 122kb long. Transgenic flies were generated by PhiC31 mediated recombination into attP2 (68A4). Injections were carried out by Duke University Model System Genomics.

To construct attB-P[acman-HA-ds+], an HA epitope tag was inserted after the signal peptide (predicted to be between Ser22 and His 23 by by UniProt (http://www.uniprot.org/) and SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) by recombineering (http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx). Briefly, a bacterial galK gene was inserted after the ds signal peptide coding sequence by amplifying galK with primers (capital letters for amplification of galK gene) ds-22S-galk-23H-F (5’-tactaattctagcatcttctggtctctgctgctca CCTGTTGACAATTAATCATCGGCA-3’), ds-22S-galk-23H-R (5’-aaggcaaagctctgcatctttgctctctctctgatg TCGACACTGTCTGCTCTT-3’).

After positive selection, the galK gene was replaced with the HA tag donor by recombination and negative selection for galK minus. The HA donor was made by hybridization and PCR with primers (capital letters for HA tag sequence) ds-22S-HA-S-23H-F (5’-tactaattctgttcttgagctcttctcttcgctgatg TCAGACACTGTCTGCTCTT-3’), ds-22S-HA-S-23H-R (5’-
Immunostaining

Wing imaginal discs were fixed in 4% paraformaldehyde in PBS followed by permeabilization in PBS with 1% BSA and 0.1% Triton X-100. Primary antibodies used for staining include rat anti-Ds (1:800, M. Simon, Stanford University) [21], mouse anti-HA (1:200, preabsorbed, Covance), rat anti-Fat (1:2000) [10], mouse anti-V5 (1:2000, preabsorbed, Invitrogen), goat anti-Wg (1:200, preabsorbed, Santa Cruz Biotechnology), rat anti-E-cad [1:200, Developmental Studies Hybridoma Bank (DSHB)], rabbit anti-Dicer2 (1:1000, Abcam), and mouse anti-Wg (1:800, DSHB). Quadruple labeling was performed using Alexa405, Cy3, and Alexa647 labeled secondary antibodies, together with the autofluorescence of Citrine (Figs1,2) or GFP (Fig. 3). Fluorescent stains were captured on a Leica TCS-SP5 confocal microscope.

Western Blotting

Wing imaginal discs were collected from third instar larvae of wild type (Oregon-R), \( ds^{36D}/ds^{UA071}, HA:ds+[attP68A] \) and \( ft^{\delta}/ft^{Grv}; HA:ds+[attP68A] \). Larvae were dissected and wing discs were separated in Ringers solution. Wing discs were lysed in RIPA buffer (50mM Tris-HCl, pH 8.0; 150mM NaCl; 1% NP-40; 0.5% Sodium deoxycholate; 0.1% SDS; 1mM EDTA; 1mM DTT and 10% glycerol, supplemented with
protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (CalBiochem)).

Primary antibodies used for staining western blot include mouse anti-HA (1:500, preabsorbed, Covance), rat anti-Ds (1:5000, M. Simon) and mouse anti-GAPDH (1:4000, Imgenex). Secondary immunofluorescent antibodies used include goat anti-mouse IRDye680 (1:10,000, Li-Cor) and sheep anti-rat IRDye800 (1:10,000, Rockland). Fluorescent detection was performed on Li-Cor Odyssey infrared imaging system and analyzed using Li-Cor software.

**Quantifying Dachs Polarity**

For the purpose of investigating regional differences in polarity, we defined distal as cells nearer to the D-V Wg stripe than to the fold that demarcates the edge of the wing pouch, we defined proximal as cells nearer to the edge of the wing pouch than to the D-V Wg stripe, and we defined dorsal hinge as cells dorsal to the fold at the edge of the wing pouch. For determining polarity as depicted in Fig. 2C, the predominant vector of Dachs polarity was estimated manually from clone borders.

For determining polarity as depicted in Fig. 5, an automated image analysis method was developed and implemented in Matlab to quantify the degree of Dachs polarity in individual cells. Figure 8A is an example of the consistent offset between Dachs and E-Cadherin staining that allows us to determine Dachs anisotropy in a cell. Quadrupally stained images as depicted in Figs 1, 2, and 5 were segmented based on E-cadherin staining to define individual cells. Some features of the data limit cell segmentation, e.g. folds in the wing disc can occur at compartment boundaries with
altered Fj or Ds expression, deforming cells to the extent that their width is comparable to the typical width of E-Cadherin staining at a membrane limiting the accuracy of recognizing cells, and confocal stacks of the wing disc capture peripodial membrane staining as well. A maximum intensity projection of the stacks will find peripodial cell membranes. An alternative projection method was developed to reduce such errors, which relies on the fact that peripodial cells are larger than epithelial cells allowing us to determine regions of an image, which have a higher density of E-Cadherin staining. This isolates regions where epithelial cells are present within each stack, which are then summed. A watershed algorithm is then implemented to recognize epithelial cell membranes. Black pixels in Figure 8A are the cell edges recognized by the algorithm.

Focusing on a single cell, as in Figure 8B, the Dachs anisotropy in the cell is apparent. To quantify this anisotropy we construct a recording region on the interior of each cell (approximately 25% of the cell size), shown in blue in Figure 8C, where each pixel's Dachs staining intensity and angle, with respect to the center of mass of the cell is recorded. Since anisotropic shapes lead to variable pixel densities, we construct mean Dachs intensities in 60 degree wedges, which are then vector summed to arrive at a final polarity vector whose orientation indicates the direction along which Dachs intensity is polarized. A typical output is shown in Figure 8C. The orientation of this vector to the tangent of the D-V Wg stripe was then estimated manually in individual cells.
FIGURES

Figure 1. Fat Signaling and Dachs Polarity in the Wing Disc

(A) Simplified schematic of the Fat signaling pathway. Fat is regulated by Dachsous and Four-jointed. Fat regulates Hippo signaling (red arrows) through Dachs and regulates PCP signaling (green arrows) partly through Dachs and partly independently of Dachs.

(B) Schematic of wing disc with the Ds gradient indicated in magenta, and the direction of Dachs polarization is indicated by green arrows. The AP and DV compartment boundaries are indicated by thin black lines, and Wg expression is indicated in red.

(C) Dachs:Cit polarization in a wing disc with WT Ds and Fj expression, stained for Wg (red), hh-Gal4 (yellow, revealed by Dcr2 staining from a UAS-Dcr2 transgene), and E-cadherin (E-cad, blue, outlines all cells). The polarity of Dachs localization is indicated by small white arrows pointing in the direction of Dachs:Cit membrane localization. Large red arrow points to the DV Wg stripe.

(D) Ds protein staining in a WT wing disc.

(E) Fj expression in a WT wing disc, revealed by a fj-lacZ transgene.
Figure 2. Nonautonomous Repolarization of Dachs by Boundaries of Fj or Ds

Expression

(A and B) Examples of wing discs with clones of cells expressing Dachs:Cit (green) from an AyDachs:Cit transgene. These discs are stained for expression of Wg (red, marks DV boundary, highlighted by large red arrow), hh-Gal4 (yellow, revealed by expression of Dcr2 from a UAS-Dcr2 transgene), and E-cad (blue, outlines cells). The polarity of Dachs localization is indicated by small arrows pointing in the direction of Dachs:Cit membrane localization; white arrows indicate normal polarity, yellow arrows indicate repolarization. Panels marked prime show the Dachs:Cit channel only from the image to the left. These animals also express either: UAS-ds (A) or UAS-fj (B).

(C) For each of four genotypes, comprising animals expressing the indicated transgenes under hh-Gal4 control, and in each of three regions (distal half of the wing pouch, proximal half of the wing pouch, or dorsal hinge), we identified anterior Dachs:Cit-expressing clones near (within eight cells) of the AP compartment boundary. In each disc, the number of cells to the farthest Dachs:Cit clone with evident repolarization toward or away from the compartment boundary was recorded (green). Because the clone frequency was relatively low, only a few relative positions are represented in each wing disc, but a representative sampling was achieved by scoring many discs (for each genotype, between 50 and 100 clones were scored in total). In addition, to set an upper limit on the extent of repolarization, we also scored the closest Dachs:Cit clones without evident repolarization (red). The range of cell numbers obtained in each case is presented using a “box and whiskers plot,” where the box indicates the range of cell distances in the middle 50% of the distribution, and the line within the box indicates the median value (if
no line is visible, the median overlapped the number of cells represented by the 25% or 75% value, and if no box is visible, it indicates that the 25% and 75% values overlapped the median). Lines outside the box extend to the maximum and minimum distances obtained in that class.

(D–G) Schematics of wing discs, with Ds, Wg, and hh-Gal4 expression indicated. Green arrows indicate direction and relative range of Dachs repolarization. See also Figure 5.
Figure 3. Polarization of Fat and Ds Localization in the Wing

(A) Example of a Ubi-GFP attB-P[acman-HA:ds+] FRT80 / attB-P[acman-ds+] FRT80 clones in a wing disc, stained for Ds (green/white) and HA (red/white) and labeled by GFP (blue) as indicated. Orange arrows indicate observed polarity of HA:Ds localization, and yellow dots mark cells at clone edges. Orange asterisk marks a cell expressing tagged Ds that has unlabeled cells on both its distal and proximal sides; this situation is rare, but when it occurs, Ds polarization is evident.

(B) Shown is the same wing disc as in (A) but at lower magnification, and with Wg expression visible (red), the white rectangle identifies the location of the image in (A).

(C) Summarized are the results of blind scoring of 73 clones for HA:Ds polarization.

(D) Shown is the same wing disc as in (F) but at lower magnification, and with Wg expression visible (red), the white rectangle identifies the location of the image in (F).

(E) Summarizes the results of blind scoring of 101 clones for V5:Fat polarization.

(F) Example of a Ubi-GFP attB-P[acman-V5:fat+] FRT80 / attB-P[acman-fat+] FRT80 clones in a wing disc, stained for Fat (green/white) and V5 (red/white) and labeled by GFP (blue) as indicated. Orange arrows indicate observed polarity of V5:Fat localization, and yellow dots mark cells at clone edges. Equal sign indicates a clone where Fat was scored as not significantly polarized. Orange asterisk marks a cell expressing tagged Fat that has unlabeled cells on both distal and proximal edges, and a slight polarization of Fat is evident.
(G) Close-up of a portion of a wing disc with a Dachs:Cit-expressing clone (green), Stained for expression of Ds (red). Panels marked by prime symbols show individual stains of the image to the left. See also Figure 6.
Figure 4. Endoproteolytic Processing of Ds

(A and B) Western blot on lysates of wing discs, from ds mutant (dsUA071/ds36D), WT, HA:Ds-expressing, and HA:Ds-expressing fat mutant (ft8/ftG-rv). The left lane contains size markers of the indicated molecular weights. The presumed identities of bands in other lanes are indicated by the labels to the right. The same membrane is depicted in both panels; (A) shows the results of anti-Ds staining, and (B) shows the results of anti-HA staining.

(C) Schematic of Ds protein. Upper indicates the approximate locations of endoproteolytic cleavage sites (red arrows) and the resulting polypeptides and the epitopes of antisera used. Ovals indicate cadherin domains, and the transmembrane domain is indicated by the thin rectangle. Lower indicates the Ds isoforms that could result from cleavage at the two different sites.

(D) Quantitation of Ds processing. The fraction of Ds in each of the three bands detected by each antisera was calculated by summing the intensities of all bands. Scale bars show the average results from six western blots on three independently prepared lysates; error bars indicate SEM. The influence of Fat on Ds processing was significant by t test evaluation of the differences between the fractions of the isoforms indicated by gray bars and asterisks (Ds-N270, p = 0.025, Ds-C210, p = 0.0008, Ds-C150, p = 0.0004). For Ds-N220, the difference was not significant (p = 0.36). See also Figure 7.
Figure 5. Nonautonomous Repolarization of Dachs by Boundaries of Fj or Ds

Expression, Related to Figure 2

(A-B) Examples of wing discs with clones of cells expressing Dachs:Cit (green) from an AyDachs:Cit transgene. These discs are stained for expression of Wg (red, marks D-V boundary, highlighted by large red arrow), hh-Gal4 (yellow, revealed by expression of Dcr2 from a UAS-Dcr2 transgene), and E-cad (blue, outlines cells). The polarity of Dachs localization is indicated by small arrows pointing in the direction of Dachs:Cit membrane localization; white arrows indicate normal polarity, yellow arrows indicate repolarization. Panels marked prime show the Dachs:Cit channel only from the image to the left. These animals also express either: A) UAS-RNAi-ds, or B) UAS-RNAi-fj.

(C-G) Rose plots to depict the vectors of Dachs:Cit polarization identified within individual anterior cells in discs of animals expressing hh-Gal4 and the indicated transgenes (C, wild-type, D, UAS-ds, E, UAS-RNAi-fj, F, UAS-RNAi-ds, G, UAS-fj). Cells were identified as distal or proximal based on their relative position within the wing pouch, and their position relative to the A-P compartment boundary was estimated by counting cells, where “1 cell” indicates a cell touching the boundary and 7 cells indicates the seventh cell from the boundary. N= the number of cells scored at that location. The diagrams are oriented with 0 at top, indicating a proximal orientation, 90 at right, indicating orientation towards the A-P boundary, 180 at bottom, indicating a distal orientation, and 270 at left, indicating orientation away from the A-P boundary. Vectors of Dachs polarization were determined using an image analysis method implemented in Matlab, as described in the methods, and the orientation of this vector to the tangent of
the D-V Wg stripe was then estimated manually in individual cells. Rose plots were generated using OSX Stereonet.
The image contains a series of diagrams illustrating the effects of various treatments on cell behavior. Each diagram represents a different condition and is labeled with the corresponding treatment:

- **UAS-ds**
- **UAS-RNAi-ds**
- **UAS-RNAi-fi**
- **UAS-fi**

Each diagram is divided into sections labeled '1 cell' to '7 cells', indicating the number of cells involved. The orientation of the cell clusters is marked as 'Proximal', 'Anterior', 'Posterior', and 'Distal', with 'N' values indicating the number of observations for each condition.

The diagrams provide a visual comparison of cell behavior across different treatments and cell counts, with arrows indicating the direction of the clusters.
Figure 6. Assaying Fat and Ds Polarity, and Its Relationship to Dachs, Related to Figure 3

(A) Schematic of the genomic region included within the P[acman-Fat+ clones.

(B) Schematic of Fat protein, the extracellular domain (ECD), Transmembrane domain (TM), intracellular domain (ICD), main cleavage site (arrow), cadherin domains (large ovals), EGF domains (small ovals), and laminG domains (pentagons) are indicated. The V5 tag is inserted at the N-terminus, just after the signal peptide.

(C) Schematic of the genomic region included within P[acman-Ds+ clones, the scale is double that shown in A.

(D) Schematic of Ds protein, the extracellular domain (ECD), Transmembrane domain (TM), intracellular domain (ICD), and cadherin domains (ovals) are indicated. The HA tag is inserted at the N-terminus, just after the signal peptide.

(E) Schematic illustration of the strategy for detecting Fat or Ds polarization. After recombination, cells heterozygous for tagged and untagged isoforms will give rise to clones of cells homozygous for tagged or untagged isoforms. The borders of clones with only untagged isoforms can then be scored for polarization of Fat or Ds by examining the staining on cells neighboring the clones.

(F) Example of a Ubi-GFP attB-P[acman-HA:ds+] FRT80 / attB-P[acman-ds+] FRT80 clones in a wing disc, stained for Ds (green/white) and HA (red/white) and labeled by GFP (blue) as indicated. Orange arrows indicate observed polarity of HA:Ds localization, yellow dots mark cells at clone edges.
(G) Example of a *Ubi-GFP attB-P[acman-V5:fat+] FRT80 / attB-P[acman-fat+] FRT80* clones in a wing disc, stained for Fat (green/white) and V5 (red/white) and labeled by GFP (blue) as indicated. Orange arrows indicate observed polarity of V5:Fat localization, yellow dots mark cells at clone edges. Equal sign indicates a clone where Fat was scored as not polarized.
Figure 7. Further Analysis of Ds Processing, Related to Figure 4

(A) Dachs:V5 staining in wild-type is polarized, and punctate.

(B) Dachs:V5 staining in ds mutant (ds^{36D}/ds^{UA071}) discs. Dachs is on the membrane, but distributed around the entire circumference of the cell.

(C) Assays of the total amount of Ds protein (obtained by summing all three bands detected) relative to a control protein (GAPDH). Bars show the average results from four Western blots on independently prepared lysates, error bars indicate sem. The influence of Fat on Ds levels was not significant by t test. (D-F) Comparison of Ds and HA staining in wing discs from animals expressing HA:Ds+ transgenes. Two small regions exhibited significant levels of Ds staining, but not HA staining, but these are not in a region where the influence of Ds on PCP has been characterized, and their significance is not known. Asterisks in D,E mark these spots where expression of Ds, but not HA, is visible.

(D) Early third instar wing disc.

(E) Late third instar wing disc.

(F) Close up of the proximal wing in a late third instar wing disc.
Figure 8. Quantifying Cellular Dachs Anisotropy

(A) Representative double staining of E-cadherin (red) and Dachs (green) wherein a slight offset can be seen in each cell. Black pixels that outline cells are a result of automated cell segmentation algorithm.

(B) Focusing on the double staining in a single cell and the apparent anisotropy.

(C) Dachs pixel intensity and orientation is evaluated in a border region on the interior of every cell, shown in blue, resulting in a Dachs polarity vector (in yellow).
CHAPTER 3

Importance of Dachs membrane localization for PCP and Hippo signaling

Abhijit Ambegaonkar, Cordelia Rauskolb and Kenneth Irvine

Results described in Figure 1 and 2 of this chapter are part of publication

Authors Contribution:
Abhijit Ambegaonkar, Cordelia Rauskolb and Kenneth Irvine conceived and designed the experiments. Results in Fig.1A, 2F-O, 3A-E were contributed by Abhijit Ambegaonkar. Results in Fig.1C-L, 2A-E were contributed by Cordelia Rauskolb.
SUMMARY

The unconventional myosin Dachs is a key effector of Ds-Fat signaling. Dachs is required for the effect of Fat on both PCP and Hippo signaling. Localization of Dachs is regulated by Fat, such that activated Fat inhibits accumulation of Dachs on membrane. However, the mechanism by which Fat affects Dachs localization, and how Fat signals downstream of Dachs was not completely understood. Here we investigate how Dachs gets polarized under the influence of Fat activity and the importance of Dachs membrane localization. Through directed membrane targeting of Dachs, we show that localization of Dachs influences both the Hippo and PCP pathways. Through in vivo live imaging and fluorescence recovery after photobleaching (FRAP) analysis; we show that Dachs is mobile on the cell membrane and Fat can influence the mobility of Dachs. Our results confirm the importance of Dachs membrane localization to downstream signaling pathways, and identify potential mechanisms for polarized localization of Dachs.
INTRODUCTION

The Dachsous-Fat pathway regulates PCP and growth to control morphogenesis (reviewed in [74, 86]). The activation of the cadherin Fat is regulated by its binding to another cadherin Ds from neighboring cell. Fj is a Golgi-localized kinase that phosphorylates cadherin domains of Fat and Ds to modulate binding between them [6, 7]. Ds and Fj are expressed in tissue wide opposing gradients, the slope of the gradients influence Hippo signaling and the direction of the gradients influence PCP [27, 74, 75]. Fat controls the localization of myosin Dachs on the membrane, such that Dachs is polarized along the Ds gradients in various tissues [1]. In absence of Fat, Dachs accumulates on all sides of the membrane whereas upon ectopic expression of Fat, Dachs is present in the cytoplasm. The amount of Dachs on the membrane correlates with the activation of Hippo signaling and the direction of Dachs polarization correlates with PCP [27, 28]. Thus, regulation of Dachs localization is important for signal transduction downstream of Fat.

The Dachsous-Fat pathway is one of the upstream regulators of Hippo pathway. The core of Hippo pathway consists of a kinase cassette, which regulates the nuclear localization of the transcriptional coactivator Yki. The Dachsous-Fat pathway regulates Hippo signaling through controlling the levels of kinase Warts (Wts) from the kinase cassette and of the FERM-domain protein Expanded (Ex) that is an upstream regulator of Hippo pathway [87, 88]. Zyxin, a LIM domain protein has been identified as a positive regulator of Yki, and is required for the effect of Fat on Hippo signaling [44]. Zyxin is localized to the subapical region on the membrane and interacts with Dachs to regulate the levels of Wts. The Ds-Fat pathway also controls the orientation of cell divisions,
which is important in determining the shape of the organ [32]. Cell division orientation is randomized in wings of dachs, fat or ds mutants, compared to the proximal-distal bias in cell division orientation in wings of wild type animals. This gives rise to rounded wings in the mutants, compared to elongated wings in the wild type animals.

Despite the observed correlation between Fat activity and Dachs localization, it was not clear how Fat influences Dachs membrane localization, and the importance of Dachs membrane localization. Here, we provide direct evidence that Dachs localization influences both Hippo and PCP phenotypes. We also investigate the dynamics of Dachs localization, which suggest that Fat can influence the mobility of Dachs on membrane.

RESULTS

Influence of directed Dachs membrane localization on Hippo and PCP signaling

To confirm the importance of Dachs membrane localization and to distinguish it from other potential influences of Fat, we sought to localize Dachs to the membrane independently of fat mutation. Studies of Zyx have identified it as a component of the Fat-Hippo pathway and suggested a model in which Dachs acts at the membrane in association with Zyx [44]. Thus, we constructed a Zyx:Dachs fusion protein, expressed under UAS control. This fusion protein exhibited a Zyx-like localization profile, as it localized to the subapical membrane around the entire circumference of the cell, rather than exhibiting the polarized localization characteristic of Dachs (Fig. 1A,B). When expressed in the developing wing under nub-Gal4 control, it resulted in a strong wing overgrowth phenotype (Fig. 1G). These overgrown wings did not flatten properly, and
hence it was difficult to compare their size with wings co-expressing wild-type forms of Zyx and Dachs, which also overgrow (Fig. 1F), but Zyx:Dachs-expressing wings nonetheless appeared to be slightly larger. A stronger activation of Yki was also evident when comparing wing discs expressing the Zyx:Dachs fusion protein with wing discs co-expressing Zyx and Dachs – the discs became more highly folded, which can be a consequence of overgrowth, and a Yki target gene, ex-lacZ, was highly expressed (Fig. 1L,L’).

The consequences of fusing Zyx and Dachs was even more dramatic when PCP was examined, as co-overexpression of Zyx and Dachs does not have significant effects on hair polarity (Fig. 2D), whereas expression of Zyx:Dachs resulted in a strong disturbance of wing hair polarity (Fig. 2E). Planar cell polarity signaling also influences the orientation of cell division, which is important in determining the shape of organs [33]. Fat-PCP signaling can polarizes cell divisions along the proximal-distal axis during wing growth, which results in elongation of the wing along the proximal-distal axis [32]. Hence, spacing between the anterior and posterior cross-veins is reduced in Fat pathway mutants. To determine the effect of Zyx:Dachs on cell division orientation, we examined the behavior of clones of GFP-marked cells in wing discs with Zyx:Dachs expressed under nub-Gal4 control. Clones were elongated along P-D axis in wild type, Zyx or Dachs expressing wing discs, but were rounded in Zyx:Dachs expressing wing discs (Fig. 2E-H, M). Cross-veins are not visible in adult wings of flies expressing Zyx:Dachs under nub-Gal4 control, which show strong overgrowth. Hence, to examine the effect of Zyx:Dachs on cross-vein distance, we have used flies heterozygous for yki^{B5} to partially suppress the overgrowth phenotype. Zyx:Dachs-expressing wings have reduced cross-
vein distance compared to wild type, Zyx or Dachs-expressing wings (Fig. 2I-L, N).
These observations indicate that Zyx:Dachs can influence cell-division orientation and
cross-vein distance. Thus, targeting Dachs to the membrane by fusing it with Zyx
phenocopies both the Hippo and PCP phenotypes of fat mutants.

**Live imaging of Dachs localization on membrane**

In epithelial cells of wing imaginal disc, Dachs has been observed to accumulate
on the distal side of the cell membrane in discrete puncta of varying size, as well as in
non-punctate continuous localization along the membrane [1]. Since Dachs is a myosin
family protein, we have tested if Dachs is mobile on the cell membrane. GFP-tagged
Dachs expressed under a genomic promoter [26] was imaged in the cells of notum
between two macrochaetes of the scutelleum region (Fig. 3A). In this region, ds and fj
forms opposing expression gradients and Dachs is polarized along the direction of
decreasing Ds expression [26], similar to wing disc. Dachs:GFP puncta were observed to
move along the membrane (Fig. 3B, 3C), and around 22% ±11% of Dachs:GFP puncta
were mobile in a cell (N = 68 puncta in 10 cells).

With these observations, we can think of several possible models for regulation of
Dachs localization and polarity towards distal side of the membrane. The observation that
Dachs can move along the membrane raise the possibility that Dachs accumulates on the
distal side of the cell through lateral mobility. Indeed, asymmetric distribution of axonal
and somatodendritic proteins to distinct domains in the plasma membrane is maintained
by influencing the lateral mobility of these proteins [89]. Moreover, since Fat is polarized
towards proximal side, it is possible that Dachs is destabilized on proximal side under the influence of Fat. Also, Dachs and Ds puncta co-localize on distal side and Dachs can physically interact with the intracellular domain of Ds [24, 26]. Hence, it is possible that Dachs is stabilized on distal side through its interaction with Ds. To investigate the influence of Ds and Fat on dynamics of Dachs localization, we examined the effect of RNAi-mediated knockdown of Ds or Fat on Dachs:GFP dynamics by Fluorescence Recovery After Photobleaching (FRAP) analysis. In this analysis, Dachs:GFP on the cell membrane of live animals was bleached with focused 488nm laser and fluorescence recovery was monitored over time. In the recovery curve, the speed of recovery indicates the mobility of the protein and the amount of protein recovered indicates the stable fraction of protein. In wild type conditions, Dachs:GFP recovered up to 60.53% its fluorescent intensity prior to bleaching, with half time of 75.22 sec (N = 9) (Fig. 3D,E). This suggests that Dachs is mobile at junctions but there was also a population of stable Dachs that did not recover. Downregulation of Fat resulted in significant decrease in speed of recovery (half time = 200 sec) compared to wild type conditions, suggesting that Dachs mobility is decreased in absence of Fat (N = 9) (Fig. 3E). Also, Dachs was recovered up to 78.42%, suggesting that stable fraction of Dachs is decreased in absence of Fat. The FRAP assay did not reveal any significant difference in recovery dynamics of Dachs after downregulation of Ds (N = 9) (Fig. 3D). Overall, the FRAP assays indicated that Fat can influence dynamics of Dachs on the membrane.
DISCUSSION

Role of Dachs localization in Fat signaling

Earlier studies identified a correlation between Dachs localization and Fat signaling, but could not prove that altered Dachs localization is a cause rather than a consequence of Fat signal transduction, nor separate the role of Dachs localization from other potential effects of Fat. We have now directly confirmed the importance of Dachs localization by creating a Zyx:Dachs fusion protein, the expression of which in otherwise wild-type animals phenocopies fat mutants both for wing growth and PCP phenotypes.

Dynamics of Dachs localization on membrane

Dachs puncta are mobile on the cell membrane, and Dachs mobility is decreased in the absence of Fat. This indicates that active Fat would influence the mobility of Dachs on the membrane such that Dachs would preferentially accumulate on the distal side of the cell. Stable fraction of Dachs was reduced in absence of Fat, which would suggest that active Fat tends to stabilize Dachs on membrane. However, there might be more components downstream of Fat and upstream of Dachs that relay the signal from Fat to Dachs. Hence, the decrease in stable fraction of Dachs in absence of Fat might be an indirect effect due to activation of Hippo signaling. We did not observe any significant change in Dachs recovery dynamics upon knockdown of Ds. This might suggest that Ds does not influence the dynamics of Dachs membrane localization. However, it is also possible that the RNAi mediated knockdown of Ds in our experiments in not sufficient enough to reduce the levels of Ds in order to have an observable effect on Dachs
dynamics. Hence, these results need to be confirmed by alternative approaches, such as conducting FRAP analysis of Dachs dynamics in *ds* or *fat* mutants, or using stronger Gal4 driver for the expression of RNAi constructs targeting *ds* and *fat*.

**MATERIALS AND METHODS**

**Drosophila genetics**

Fly stocks used for investigating the effect of Zyx:Dachs fusion protein on PCP and Hippo signaling included:

*UAS-dcr2[X]; nub-Gal4 [ac-62]*

*UAS-Zyx:V5/TM6B*

Fly stocks used for investigating the dynamics of Dachs localization and FRAP analysis include:
w; d:GFP [VK19] ubi-Baz:mCherry/TM6b

w; pnr-Gal4 UAS-dcr2/TM6b

y w hs-FLP[122]; UAS-RNAi-ds[vdrc36219]; d:GFP [VK19]/TM6b

y w hs-FLP[122]; UAS-RNAi-fat[vdrc9396]; d:GFP [VK19]/TM6b

Flip-out clones of Zyx:Dachs were induced by heat shock (33°C for 20 min) during early third instar, and larvae were fixed at mid-third instar. Flip-out clones of GFP were induced by heat shock (33°C for 20 min) during second instar, and larvae were fixed at mid-third instar.

To quantify cross-vein distance, the length of vein L4 between cross-veins was measured using ImageJ, and divided by the length of vein L3, to get a relative length, and these were normalized to the wild-type ratio. To quantify clone shape anisotropy, borders of GFP expressing clones were marked in ImageJ and an ellipse was fitted into the shape of the clone. Elongation ratio was calculated as ratio of major axis to minor axis.

**Plasmids and Constructs**

Zyxin was amplified by PCR from pUAST-attB-Zyx102.44:V5 [44] using forward primer (5’ - TGAATAGGAATTGGGACCATGGAGTCTGTGGGCCAGCAACTTAG - 3’) and reverse primer (5’ - CATGGCGGCGGCCAGATGTTCTGACGTCATGCGGTTTG - 3’). Dachs was amplified by PCR from pUAST-attB-d:V5 [1] using forward primer (5’ - CTGGCCGCGCCATGTTGACTACGACGATCTGGACAGATTG - 3’)} and reverse
primer (5’ -
CCTTCACAAAGATCCTTTACGTAGAATCGAGACCGAGGAGAGGGTTAG -3’).
The Zyxin and Dachs fragments were inserted using InFusion cloning kit into pUAST-attB after digestion with EcoRI and XbaI, to generate pUAST-attB-zyx-LAAA-d:V5.

**Immunostaining**

Wing imaginal discs were fixed in 4% paraformaldehyde in PBS followed by permeabilization in PBS with 1% BSA and 0.1% Triton X-100. Primary antibodies used for staining include mouse anti-V5 (1:2000, preabsorbed, Invitrogen), rat anti-E-cad [1:200, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Wg (1:800, DSHB) and goat anti-β-galactosidase (1:1,000, Biogenesis). Fluorescent stains were generated using Alexa488, Cy3, and Alexa647 labeled secondary antibodies and captured on a Leica TCS SP5 confocal microscope.

**Live imaging and fluorescence recovery after photobleaching (FRAP) analysis**

Notum of pupae (16 h after white prepupa formation) were prepared for live imaging as described in [90]. For investigating the dynamics of Dachs:GFP puncta, cells of notum were imaged on Perkin Elmer Ultraview spinning disc confocal microscope. Dachs:GFP was imaged using 488 nm laser (output 25%) and 500-550 nm bandpass filter. Baz:mCherry was imaged using 561 mn laser (output 30%) and 582-618 nm bandpass filter. Images were taken after every 10 seconds for 20 minutes. For FRAP analysis, Dachs:GFP in cells of notum was bleached using 488 nm laser (output 100 %) with 25 passes over the region of interest (ROI). Three pre-bleach and 80 post bleach
images were taken after every 15 seconds. Mean fluorescence of the ROI was quantified using Volocity and one phase exponential association curve was fitted on the data after plotting in Prism (v.5 GraphPad).
FIGURES

Figure 1. Influence of Zyxin-Dachs fusion protein on wing growth

(A and B) Portions of wing imaginal discs with clones of cells expressing Zyx:Dachs:V5 (green) in (A) horizontal or (B) vertical section.

(C-G) Adult wings from animals expressing (C) nub-Gal4 and (D) UAS-Zyx, (E) UAS-dachs, (F) UAS-Zyx UAS-dachs or (G) UAS-Zyx:dachs.

(H-L’) Wing discs from animals expressing (H) en-Gal4 (marked by UAS-GFP, green) and (I) UAS-Zyx, (J) UAS-dachs, (K) UAS-Zyx UAS-dachs or (L) UAS-Zyx:dachs, stained for ex-lacZ (red). (H’-L’) ex-lacZ channel only.
Figure 2. Influence of Zyxin-Dachs fusion protein on PCP

(A-E) Anterior proximal wing from animals expressing (A) nub-Gal4 and (B) UAS-Zyx, (C) UAS-dachs (D) UAS-Zyx UAS-dachs or (E) UAS-Zyx:dachs.

(E-H) Wing imaginal discs from animals expressing (E) nub-Gal4 and (F) UAS-Zyx, (G) UAS-dachs or (H) UAS-Zyx:dachs.

(I-L) Adult wings from animals heterozygous for yki\textsuperscript{B5} along with expressing (I) nub-Gal4 and (J) UAS-Zyx, (K) UAS-dachs or (L) UAS-Zyx:dachs. Black arrows indicate the position of cross-veins.

(M) Quantification of clone shape anisotropy for clones in E-H.

(N) Quantification of cross-vein distance for adult wings in I-L.
**Figure 3. Mobility of Dachs-GFP puncta on membrane and FRAP analysis of Dachs-GFP**

(B and C) Time points from images (B: 0 sec and B: 600 sec) indicating the change in position of tracked Dachs:GFP puncta (green) indicated by white arrow, relative to cell membrane marked by Baz:mCherry (red).
CHAPTER 4

Coordination of PCP signaling pathways through Spiny legs

Abhijit Ambegaonkar and Kenneth Irvine

Results described in this chapter are under revision for publication in eLife.
SUMMARY

Morphogenesis and physiology of tissues and organs requires planar cell polarity (PCP) systems that orient and coordinate cells and their behaviors, but the relationship between PCP systems has been controversial. We have characterized how the Frizzled and Dachsous-Fat PCP systems are connected through the Spiny-legs isoform of the Prickle-Spiny-legs locus. Two different components of the Dachsous-Fat system, Dachsous and Dachs, can each independently interact with Spiny-legs and direct its localization in vivo. Through characterization of the contributions of Prickle, Spiny-legs, Dachsous, Fat, and Dachs to PCP in the Drosophila wing, eye, and abdomen, we define where Dachs-Spiny-legs and Dachsous-Spiny-legs interactions contribute to PCP, and provide a new understanding of the orientation of polarity and the basis of PCP phenotypes. Our results support the direct linkage of PCP systems through Sple in specific locales, while emphasizing that cells can be subject to and must ultimately resolve distinct, competing PCP signals.
INTRODUCTION

Planar cell polarity (PCP) is the coordinated orientation of cell structures and behaviors within the plane of a tissue. Manifestations of PCP include the orientation of hairs, bristles, stereocilia, and ommatidia, as well as orientated cell divisions and cell movements. Two conserved molecular systems play key roles in the establishment and maintenance of PCP: the Frizzled (Fz) PCP pathway and the Dachsous (Ds)-Fat PCP pathway [76, 91]. These involve distinct components, but share common features, including the polarized localization of key components within cells, and the existence of asymmetric intercellular interactions that enable this polarity to be propagated from cell to cell. Many processes are influenced by both PCP systems, but the relationship between them has been controversial.

The *Drosophila* Ds-Fat PCP pathway includes the cadherin family proteins Ds and Fat, which interact between neighboring cells [4, 30, 61]. Binding between Ds and Fat is modulated by Four-jointed (Fj), a Golgi localized kinase that phosphorylates their cadherin domains [5-7]. Ds and Fj are expressed in opposing gradients, which orient Ds-Fat PCP [1, 20-23, 30]. Fat protein in a cell within a Ds-Fj gradient preferentially accumulates along the side where it contacts cells with higher Ds and lower Fj; Ds protein localizes in a complementary orientation (Fig. 1A) [24-26]. How polarization of Ds and Fat proteins establishes PCP is incompletely understood, but it is achieved in part through the unconventional myosin Dachs, whose membrane localization is regulated by Fat [1]. Mammalian homologues of Ds and Fat, Dchs1 and Fat4, also influence PCP [34-36], and human FAT4 can rescue PCP phenotypes of *Drosophila* fat mutants [12].
The *Drosophila* Fz PCP pathway includes the asymmetrically distributed transmembrane proteins Fz and Van Gogh (Vang, also known as Strabismus), which act together with the cadherin family protein Starry night (Stan, also known as Flamingo) (Fig. 1A) [47, 50, 76, 92-94]. Stan interacts with Stan-Fz heterodimers in neighboring cells [55, 95, 96]; interactions between Vang and Fz have also been reported [97]. Each of these transmembrane complexes is associated with distinct cytoplasmic proteins, Fz-Stan associates with Dishevelled (Dsh) and Diego (Dgo) [48-51]. Vang-Stan associates with Prickle-Spiny legs (Pk-Sple) [52, 53]. Polarization of these protein complexes can propagate from cell to cell through heterophilic intercellular interaction between Vang-Stan and Fz-Stan complexes [55, 76, 95]. Polarization of complexes within a cell is reinforced by inhibitory intracellular interactions between asymmetrically localized components [76]. Mutation of any of the core members of the Fz PCP system will impair the polarization of all of the others, emphasizing their mutual dependency for polarization [80].

Genetic interactions between Ds-Fat pathway genes and Fz pathway genes, together with observations of altered Fz pathway protein localization in Ds-Fat pathway mutants, led to suggestions that the Ds-Fat pathway acts upstream of the Fz pathway [21, 61]. According to this hypothesis, the Ds-Fat pathway acts as a "global module" that provides long-range directional information through tissue-wide Fj and Ds gradients, whereas the Fz pathway acts as a "core module" that establishes robust polarization that can propagate locally, and effects cellular polarity. This suggestion was challenged by observations that clones of cells mutant for or over-expressing *ds, fj* or *fat* in the abdomen can affect PCP non-autonomously even in the absence of Fz pathway components [63].
Additionally, in the abdomen, combining mutations in both Ds-Fat and Fz pathway genes can have more severe effects on PCP than single mutants, suggesting that these pathways can act in parallel [63, 64, 98]. There are also some manifestations of PCP, such as oriented cell divisions in the developing wing, which are influenced by the Ds-Fat pathway and not the Fz pathway [33].

Nonetheless, other studies have provided evidence of cross-talk between PCP systems, and implicated the Pk-Sple locus in helping to mediate this cross-talk. The Pk-Sple locus produces two functional isoforms: Prickle (Pk) and Spiny legs (Sple), which share a common, LIM-domain containing C-terminus, but unique N-termini (Fig. 1 supplement l) [57]. These isoforms have distinct roles: for example, mutations that only affect pk disrupt PCP in the wing and notum, but not in the eye and leg, whereas mutations that only affect sple exhibit a complementary specificity. The observations that mutations that affect both isoforms (pk-sple) have milder effects on PCP than isoform-specific alleles in the wing, notum, and leg, and that over-expression of Sple or Pk leads to PCP phenotypes reminiscent of loss-of-function of pk, or sple, respectively, led to the suggestion that a balance between Pk and Sple isoforms is necessary for normal PCP [57]. Studies of PCP establishment in the pupal wing revealed that it occurs in distinct phases, with an early phase controlled by the Ds-Fat pathway in a process that depended specifically upon the Sple isoform [62, 99]. Moreover, examination of PCP protein localization revealed that a coupling between the polarization of components of the Ds-Fat and Fz systems could be induced by expression of Sple [99]. Additionally, in the abdomen, the Fj and Ds gradients are oriented oppositely within anterior (A) versus posterior (P) compartments of each segment [22]. Since hairs always point posteriorly,
this led to the suggestion that there could exist a "rectification" mechanism, which would reverse the influence of these gradients on hair polarity. The observations that Sple over-expression reverses polarity in P compartments, and that pk-sple mutants reverse polarity in part of the A compartment, led to the suggestion that Pk and Sple might be involved in this rectification [100].

Two potential mechanisms by which Pk-Sple might influence the relationship between PCP pathways have recently been suggested. It was reported that Dachs could directly interact with Pk and Sple, and that Ds and Dachs could influence Sple localization in wing discs [60]. It has also been proposed that Pk-Sple could connect PCP pathways through an influence on microtubule orientation [59]. Vesicles containing Fz and Dsh have been observed to move along apical non-centrosomal microtubules towards the distal side of wing cells, with the proximal-distal alignment of microtubules and consequent directional transport of Fz pathway components dependent upon the Ds-Fat pathway [59, 65-67]. Pk and Sple also influence the orientation of apical microtubules, such that the plus ends of microtubules are preferentially found at either the high end or the low end of the Ds gradient, depending on whether Pk or Sple, respectively, is the predominant isoform [59, 67]. Relative differences in expression of isoforms consistent with their distinct requirements have also been reported: Pk at higher levels than Sple in larval wing discs, and Sple at higher levels than Pk in eye discs [59, 60, 99]. While these studies are suggestive of a key role for Pk-Sple in linking PCP pathways, the extent to which these or other mechanisms link PCP pathways, and their contribution to orienting PCP, remain unclear.
Here, we demonstrate that Dachs and Ds can each physically interact with Sple, and control its localization in the wing, eye and abdomen. Our studies complement observations of Ayukawa et al. (2014) in identifying requirements for Dachs and Ds in Sple localization, but differ regarding the nature of these requirements. We also extend understanding of the relationship between Ds-Fat and Fz PCP pathways by identifying organ and region-specific differences in their interactions, and illustrate how this relationship between pathways can explain poorly understood features of PCP mutant phenotypes. Our results establish control of Sple localization as a key mechanism by which the Ds-Fat pathway coordinates with Fz to influence PCP, and enhance our understanding of how PCP is coordinated in developing tissues.
RESULTS

Distinct localization of Pk and Sple in wing imaginal discs

Components of each of the two major PCP pathways are polarized along the proximal-distal axis of the larval wing imaginal disc and pupal wing (Fig. 1A) [76, 91]. Components of both pathways are required for the normal distal orientation of wing hairs. Motivated by observations implicating the pk-sple locus in modulating the influence of the Ds-Fat pathway on wing hair and ridge polarity [62], we initiated experiments to examine the localization of the distinct Pk and Sple isoforms and their potential regulation by Ds-Fat PCP. This was achieved by expressing GFP-tagged isoforms in clones of cells. Consistent with recent studies examining isoform-specific localization [60, 101, 102], we observed that GFP:Pk was polarized towards the proximal sides of cells, except just anterior to the anterior-posterior compartment boundary, where GFP:Pk was instead polarized towards the anterior sides of cells (Fig. 1D,G, 2I). By contrast, GFP:Sple was polarized towards the distal sides of cells throughout the wing disc (Fig. 1C,E,F, 4I). The distinct localization of Pk and Sple expressed in wing discs indicates that they can respond to distinct spatial cues.

Dachs and Ds can physically interact with Sple

The localization of Sple to the distal side of wing disc cells is similar to that of Dachs and Ds (Fig. 3) [1, 24, 25, 27]. To investigate whether this shared localization
could reflect physical association, we assayed for co-immunoprecipitation of epitope-tagged proteins expressed in cultured *Drosophila* S2 cells (Fig. 2). Indeed, V5-tagged Dachs could co-immunoprecipitate Flag-tagged Sple (Fig 1B, lane 1). Dachs and Sple interact through the unique N-terminus of Sple, because Dachs also co-precipitated a construct comprising only the Sple N-terminus (Sple-N), but did not co-precipitate a full length Pk construct (Fig. 1B, lanes 2 and 3). Interaction with Ds was investigated by expressing a construct comprising the entire intracellular domain of Ds (Ds-ICD). Both Sple and Sple-N also interacted with Ds-ICD, whereas Pk did not (Fig. 1B, lanes 4-6).

We note that Ayukawa et al. [60] similarly reported an ability of Dachs to interact with Sple. However, our results differ in that they reported that Dachs could also interact with Pk, whereas we could not detect any interaction between Pk and Dachs above non-specific background (defined by precipitation observed using GFP:V5 instead of Dachs:V5, Fig. 1B lane 11). Also, Ayukawa et al. reported that they could not detect an interaction between DsICD and full length Sple, leading them to suggest a requirement for other components such as Dachs, whereas we did detect this interaction (Fig. 1B, lane 4). Altogether, our results establish that Dachs and Ds can each independently interact with Sple, and that they do so through its unique N-terminal region.

**Influence of Dachs and Ds on Sple localization in wing discs**

To determine whether the shared distal localization and physical interaction between Dachs and Sple are reflective of a functional role for Dachs in localizing Sple, we examined GFP:Sple in *dachs* mutant wing discs. Indeed, GFP:Sple localization was
altered, as throughout most of the developing wing disc its localization became similar to that of Pk: on the proximal side of cells, and in fewer, more discrete puncta (Fig. 4A,I). Along the A-P compartment boundary, GFP:Sple was instead localized towards anterior side of cells, as is GFP:Pk (Figs 4I, 5A) [101]. Intriguingly, however, in the most proximal part of the wing pouch, GFP:Sple generally maintained a distal localization (Fig. 4B, I). Thus, Dachs is required for the distal localization of GFP:Sple throughout most of the wing pouch, but not in the proximal wing.

In ds mutant wing discs, GFP:Sple was either unpolarized (localized to cell membrane on all sides, 51% of clones, N=190), partially polarized (on multiple cell sides but with a clear bias, 28% of clones), or polarized in random directions (21% of clones) (Fig. 4D,I). The observation of unpolarized GFP:Sple is consistent with the inference that Dachs can localize GFP:Sple, because Dachs is localized to all membranes in an unpolarized fashion in ds mutants [24]. Dachs is similarly localized to the membrane in an unpolarized fashion in fat mutants [1] (Mao et al., 2006), and we always observed unpolarized membrane localization of GFP:Sple in fat mutant wing discs (Fig. 4C). To confirm that mis-localization of Dachs is responsible for the mis-localization of GFP:Sple in ds or fat mutant wing discs, we examined GFP:Sple in ds dachs and fat dachs double mutants. In both cases, GFP:Sple reverted to a Pk-like localization, including a proximal, punctate orientation throughout most of the wing pouch, and an anterior orientation near the A-P boundary (Fig. 4E-I).

One remarkable feature of ds dachs or fat dachs mutant discs is that GFP:Sple localization is preferentially proximal even in proximal regions of the wing pouch, where GFP:Sple localization was preferentially distal in dachs mutants (Fig. 4B,F,H,I). This
implies that the distal localization of GFP:Sple here in *dachs* mutants is Ds-dependent, and hence that Dachs and Ds each have the ability to independently localize GFP:Sple. Thus, in proximal cells, where Ds expression is higher, Dachs and Ds could provide redundant localization cues for GFP:Sple. In distal cells, by contrast, we suggest that Dachs could be required for GFP:Sple localization because Ds expression is too low. In *ds* or *fat* mutants, Dachs is mis-localized, and Ds is either absent (in *ds* mutants) or unpolarized with reduced junctional accumulation (in *fat* mutants) [8], and consequently GFP:Sple becomes mis-localized. Finally, in the absence of both Dachs and Ds localization cues, as in *fat dachs* or *ds dachs* mutants, GFP:Sple follows Pk localization cues. In principle this could occur either because Sple is able to respond directly to the same cues as Pk through shared motifs, or because Sple can bind to Pk. The observation that GFP:Sple localized proximally in *dachs pk* mutant wing discs (Fig. 5F) indicates that Sple can respond to Pk localization cues even in the absence of Pk. Localization of Pk was not visibly altered within larval wing discs by *dachs, ds, or fat* mutations (Fig. 5).

We note that our studies agree with Ayukawa et al. (2014) in reporting an influence of *dachs* and *ds* on Sple localization, but differ in that they reported that in *ds* mutants Sple polarity was reversed, resembling Pk, whereas we observe either a complete absence, or a randomization, of Sple polarization in *ds* mutants, consistent with Sple being regulated by Dachs. Also, they did not report observing the difference in localization of Sple between distal and proximal regions of *dachs* mutants, which we determined reflects a Dachs-independent regulation of Sple by Ds. Nonetheless, our studies agree that a direct connection between the Fz and Ds-Fat PCP pathways can be mediated through Dachs and Ds-dependent control of Sple.
PCP in pk mutant wings reflects Dachs-directed polarity

The link between PCP pathways mediated through Sple has important implications for how PCP is oriented, and suggests explanations for the basis of both pk and fat mutant polarity phenotypes. sple mutation does not result in a hair polarity phenotype in the wing, whereas pk mutants have a characteristic wing polarity phenotype, in which hairs in much of the wing are mis-oriented away from the wing margin, and wing margin bristles can point proximally (Figs 7D,H) [57]. The observation that Pk and Sple can localize in opposite directions, whereas wing hairs normally point in a single direction, implies that cells must ultimately choose which of these two distinct localization cues to follow. Normally, they choose the Pk cue (Fig. 6A), presumably because the Pk isoform is more abundant than the Sple isoform in the wing [59, 60, 99], and hence it dictates polarization. Indeed, if Sple is over-expressed, then hair polarity is reversed even more strongly than in pk mutants, and can align with Ds-Fat PCP [57, 59, 60, 99, 103]. These observations suggest that wing hair polarity in pk mutants could be directed by the Ds-Fat pathway dependent polarization of Sple (Fig. 6A). As this linkage in most of the wing depends upon dachs (Fig. 4I), this hypothesis predicts that the pk wing hair polarity phenotype should be suppressed by dachs mutation (Fig. 6A). Indeed, when we tested this by comparing wing hair and bristle orientation in pk versus dachs pk mutants, this suppression was observed (Fig. 6C-J). This result can also explain the observation that over-expression of Fat could suppress the pk hair polarity phenotype [62], because over-expression of Fat removes Dachs from the membrane [1], which, as Dachs functions at membranes [12, 44], is functionally equivalent to dachs mutation.
Sple contributes to Fat PCP phenotypes in the wing

The determination that Ds-Fat and Fz pathways are molecularly linked by physical interaction between Dachs and Sple also provides a new perspective on polarity phenotypes of fat and ds. The altered wing hair polarity in fat or ds mutants has been interpreted as indicating that Fat and Ds have a normal role in directing hair polarity in regions of the wing. Indeed, recent studies have inferred that Ds-Fat PCP influences core protein polarization in the wing by orienting microtubules [59, 65, 67]. However, as hair polarity in the wing is normally Pk-dependent rather than Sple-dependent, and as we found that Ds-Fat PCP in the wing influences Sple localization but not Pk localization, we considered an alternative model: rather than reflecting a normal role in directing hair polarity, these phenotypes of fat and ds could stem from the inappropriate accumulation of Dachs, leading to inappropriate localization of Sple, which in some contexts could interfere with the normal Pk-dependent polarization cues (Fig. 6A). Consistent with this hypothesis, we confirmed that fat wing hair polarity phenotypes (generated using wing-specific RNAi) are suppressed by dachs (Fig. 7). This hypothesis further predicts that fat PCP phenotypes could be suppressed by sple (Fig. 6A), and while we did not observe completely normal hair polarity in fat sple wings, we did observe a partial suppression, including restoration of normal, distally oriented polarity in two regions affected by loss of Fat: near the proximal anterior wing margin, and near the anterior cross-vein (Figs 7B,K-N). Thus, while Sple is not required for normal wing hair polarity, it mediates a connection between Ds-Fat and Fz pathways that contributes to abnormal hair polarity in the absence of fat.
Control of Sple polarity in eye discs by Ds-Fat PCP

PCP in the eye has been studied for its influence on the organization and orientation of ommatidia [104]. The eight photoreceptor cells within each ommatidia are arranged in a characteristic pattern that comes in two chiral forms. This chirality is determined by which of two neighboring photoreceptors becomes the R3 cell and which becomes R4. This decision is dependent upon Notch signaling, which is biased by Fz PCP such that the cell at the R3-R4 interface with higher Fz becomes R3 [30, 70, 71, 105]. The two chiral forms are established in mirror symmetry with respect to the dorsal-ventral compartment boundary, termed the equator (Fig. 9). In sple mutants, ommatidial chirality is randomized, whereas in pk mutant eyes ommatidial chirality is normal [57]. Ds and Fj are expressed in complementary gradients in the eye (Fig. 9), and experiments manipulating Ds and Fj expression have revealed that these gradients instruct normal polarity [20, 21, 30, 106]. However, the relationship between Fz and Ds-Fat PCP pathways in the eye and how this influences polarity has remained unclear. Also, in contrast to the wing and abdomen, where dachs mutation suppresses fat PCP phenotypes, dachs mutation has little effect on fat PCP phenotypes in the eye [1, 25, 107]. We hypothesized that the influence of Ds-Fat PCP on ommatidial polarity might be accounted for by an ability of Ds to polarize Sple independently of dachs, as in the proximal wing.

Ommatidia form progressively in a wave of differentiation that sweeps across the eye disc, initiated within a line of cells that form the morphogenetic furrow. We analyzed
GFP:Sple localization at the 5-cell precluster stage of ommatidial formation, when R3-R4 specification occurs. GFP:Sple localized to the equatorial side of cells within both R3 and R4, which places it within R4 at the R3-R4 interface (Fig. 8A). Equatorial polarization of Sple co-localizes it with Vang [51], and is consistent with the observation that it interacts with Ds, since Ds is also polarized to the equatorial side of cells in the eye disc [25]. This equatorial polarization of Sple was disrupted in ds or fat mutants (Fig. 8C,E), but was not affected by mutation of dachs (Fig. 8B), nor could dachs mutation prevent the mis-localization of Sple in ds or fat (Fig. 8D,F). In ds or fat mutants, Sple localization was partially randomized within R3 and R4, and also partially unpolarized, in that it was often detected on multiple cell junctions. However, it was never detected along the cell junction with the more anterior cells within the ommatidial cluster (R2 and R5) (Fig. 8).

We also examined localization of GFP:Pk within R3 and R4, and found that it too localized to the equatorial side of both cells (Fig. 8G). How might Pk localize equatorially if it cannot interact with Ds? We hypothesized that this might arise from an ability of Pk-Sple proteins to multimerize, or from the interactions that lead core components of the Fz pathway to adopt a shared, discrete, polarized localization. In such cases, equatorial polarization of Pk would not come about because it directly responds to an equatorial-polar signal like Ds, but rather because it can interact with Sple and/or Vang, which recruit it to equatorial cell junctions. In support of this, we found that in sple mutants, Pk localization is altered such that it becomes partially randomized, resembling Sple localization in fat or ds mutants (Fig. 8H). Thus, equatorial Pk localization in R3 and R4 depends upon Sple.
The posterior bias in Pk localization in *sple* mutants, and the similar posterior bias in Sple localization within *ds* or *fat* mutants, tend to place Pk or Sple towards the side of the cell nearest the morphogenetic furrow (Fig. 8). The morphogenetic furrow is a source of local signals for multiple pathways, including Notch, Hedgehog and Decapentaplegic, which might, indirectly at least, influence Fz PCP orientation in these mutant eye discs, as they have been implicated in influencing PCP in other contexts [72, 101]. Finally, we observed that in front of the morphogenetic furrow, GFP:Sple and GFP:Pk exhibit distinct localization profiles, with GFP:Sple accumulating on the equatorial sides of cells, as it does behind the furrow, but Pk:GFP accumulating on the anterior sides of cells, which in this region is the side closest to the morphogenetic furrow (Fig. 10). Thus, behind the morphogenetic furrow, Sple is localized by Ds, and Pk is localized by Sple, whereas in front of the morphogenetic furrow, Sple and Pk can localize independently.

**Interactions between PCP pathways in the abdomen**

Hairs in the *Drosophila* abdomen point posteriorly; this orientation is influenced by components of both the Fz and Ds-Fat PCP pathways [22, 63, 100]. In analyzing the relationship between PCP pathways in the abdomen, we focused on the pleural cells, which form in lateral and ventral regions, but have also examined polarity in tergites, which form on the dorsal side of the abdomen. As the subcellular localizations of Dachs, Sple and Pk within pupal abdominal cells have not been described, we first characterized their distributions in pleural cells of wild-type animals at pupal stages, with posterior compartments marked using *hh-Gal4* and *UAS-RFP* transgenes. Dachs:GFP and
Sple:GFP were polarized towards the anterior sides of cells within A compartments, and towards the posterior sides of cells within P compartments (Fig. 11A,B,H). This is consistent with their being polarized in response to the Ds and Fj gradients, as the Fj and Ds gradients are oriented oppositely within anterior (A) versus posterior (P) compartments of each segment (Fig. 12) [22], and Dachs and Sple accumulate on the sides of cells that face towards lower Ds levels and higher Fj levels. Pk:GFP, by contrast, was polarized towards the anterior sides of cells within both A and P compartments (Fig. 11C,H). Thus in A compartments Pk:GFP and Sple:GFP polarize in the same direction, whereas in P compartments they polarize in opposite directions.

Consistent with prior studies [100], we observed that pk-sple mutants reverse hair polarity within the center of the A compartment, while the P compartment, and the edges of the A compartment, exhibit normal hair polarity (Fig. 13B,F). We then extended analysis of pk-sple by examining isoform-specific alleles. pk mutant alleles have normal polarity in A compartments, but mostly reversed polarity in the P compartment within pleura (Fig. 13C), although not in tergites (Fig. 13G). sple mutant alleles have normal polarity in the P compartment, and abnormal polarity, including hair reversal but also sideways or swirling hair orientations, within the center of the A compartment (Fig. 13D,H).

The influence of pk-sple on polarity in the P compartment of the pleura thus appears reminiscent of the situation in the wing: Pk and Sple can respond to opposing localization cues. In the absence of Pk, cells respond instead to Sple-dependent cues, leading to a reversal of polarity (Sple localized normally in pk mutants, Fig. 14I,K). The essential contribution of Sple to the pk phenotype is confirmed by its suppression in pk-
sple mutants (Fig. 13). The influence of Pk-Sple on polarity in A compartments is reminiscent of the situation in the eye. Sple, not Pk plays the key role in establishing polarity here, and Pk was mis-localized within the central region of A compartments of sple mutants (Fig. 11G,H), hence Sple contributes to localization of Pk here. In fat or ds mutants, hair polarity is disturbed in much of the A and P compartments, although a small region at the front of the A compartment exhibits normal hair polarity (Figs 15A,E, 16A,E) [22]. To determine whether the abnormal polarity could be explained by mis-localization of Dachs and/or Ds, and a consequent mis-localization of Sple and/or Pk, we assessed both genetic interactions and protein localization. The disruption of polarity within A compartments in fat or ds mutants was correlated with mis-localization of Dachs throughout the A compartment (mostly uniform Dachs in fat mutants, and randomized Dachs in ds mutants, Figs 11D, 14A,K), and mis-localization of Sple everywhere except the most anterior region of the A compartment (Fig. 11E,H, 14B). Moreover, mutation of dachs suppressed the hair polarity phenotypes of fat and ds in A compartments (Figs 15 C,F, 16 C,G) [1], and also suppressed the mis-localization of Sple (Figs 11F,H, 14D). These observations suggest that ds and fat polarity phenotypes in the anterior abdomen can be accounted for by a Dachs-dependent mis-localization of Sple, as we had observed in the wing. Mutation of dachs alone does not disrupt polarity in A compartments (Figs 15B, 16B), perhaps because Ds is sufficient to direct Sple localization. Alternatively, it could be that in the absence of Dachs, Sple is localized by the same cues that localize Pk, as Pk localization remained normal within A compartments of ft or ds mutants (Fig. 14E,F,K).
In P compartments, Dachs is mis-localized in fat or ds mutants, and there is also a partial mis-localization of Sple (Figs 11,14). However, mutation of dachs alone causes a reversal of hair polarity in P compartments (Figs 15B, 16B) [17]. This reversal of polarity is associated with a reversal of Pk localization (Fig. 14G,K). The P compartment of the abdomen thus differs from other regions we have examined both in that there is a strong PCP phenotype associated with mutation of dachs, and in the mis-localization of Pk in dachs mutants. One potential explanation for this could be that in the absence of Dachs, Pk localization becomes governed by Sple, which retains its normal posterior localization in dachs mutants (Figs 11G, 14C). In dachs sple or dachs pk mutant abdomens, there is still some reversal of polarity in P compartments, although the region of reversal appears narrow than in dachs mutants (Fig. 16F,H). Pk localization is also disturbed in P compartments of fat or ds mutants (Fig. 14E,F,K), as well as in fat dachs or ds dachs double mutants (Fig. 14H,J,K). Thus, while there are some similarities in control of PCP between abdominal P compartments and wings, there are also differences, hence distinct mechanisms contribute to the control PCP in each of these body regions.
DISCUSSION

**Sple can link PCP pathways**

Our results implicate interactions between Dachs and Sple, and between Ds and Sple, as a connection point between PCP pathways coordinating polarity in multiple *Drosophila* organs. We found that both Dachs and Ds can each independently bind to Sple, but not Pk, through the unique N-terminus of Sple. Dachs and Ds also each have the ability to influence Sple localization, and we identified some places where Dachs is necessary and sufficient to localize Sple (i.e. distal wing), and others where Dachs is dispensable but Ds is required (e.g. proximal wing, eye). The influence of Ds on Sple localization is essential for some manifestations of PCP, such as ommatidial polarity in the eye. Indeed, because Fz PCP can propagate from cell to cell, we propose that localization of Sple by Ds could account for the long range influence of Ds-Fat borders on PCP in the eye [21, 30, 107]. The influence of *dachs* on PCP is generally mild, likely due to partial redundancy with Ds in localizing Sple. However, a strong influence of Dachs is revealed in the context of additional PCP mutations, as it contributes to the disturbed Sple localization and hair polarity in the wing and abdomen of *fat* and *ds* mutants, as well as the reversal of wing hair polarity in *pk* mutants.

Several mechanisms by which Pk-Sple could influence the polarization of components of the Fz-PCP system have been described. These include physically associating with Vang, and promoting clustering, endocytosis, and/or degradation of Vang or other PCP proteins [52, 56, 102, 108, 109]. They have also been identified as
influencing the orientation of apical non-centrosomal microtubules that can traffic components of the Fz-PCP system, including Fz and Dsh [59, 67]. The various activities ascribed to Pk-Sple are not mutually exclusive, and it could play multiple roles. However, the only unique functions clearly attributed to Sple as opposed to Pk are its interactions with Ds and Dachs, and distinct localization. Fz and Fat localize to opposite sides of cells in wings, but to the same side in eyes [91] (Fig. 1A, 9). Because Sple expression is relatively high in eye and low in wing [59, 60, 99], our studies are consistent with a molecular explanation for "rectification" of this relationship between PCP pathways in which interaction of Sple with Dachs and Ds links PCP pathways in eyes but not in wings; coupling between pathways that depends upon physical interactions with Sple and not Pk could similarly explain the opposite relationships between hair polarity and Ds and Fj gradients in A versus P abdominal compartments. Thus, the solution our results support for the controversy over the relationship between the two PCP pathways is that in some contexts they operate in sequence, with directional information passed from Ds-Fat PCP to Fz PCP via Sple, whereas in other contexts they are uncoupled.

While vertebrates have a Ds homologue that is required for PCP, Dchs1 [35], Dchs1 must influence PCP in mammals through a distinct mechanism, as Pk is conserved in vertebrates, but the Sple isoform is not. Even in flies, the linkage of Dachs and Ds to Sple cannot be the sole mechanism by which Ds-Fat signaling influences PCP, as some manifestations of cell polarity controlled by Ds-Fat, e.g. oriented cell divisions, do not require Sple [33, 57]. Moreover, when mis-expressed, Ds and Fat can alter PCP even in flies lacking a functional Fz PCP system (e.g. fz- stan- flies) [63]. It has also been proposed that PCP in the wing is influenced by shear forces generated by contraction of
the wing hinge [110], and disruption of these shear forces in fat or ds mutants might occur through a mechanism that depends upon dachs but not sple, due either to effects of Dachs on Hippo signaling [43] or on cytoskeletal tension [26, 32]. Such additional influences of Dachs might explain why fat wing hair polarity phenotypes are more strongly suppressed by loss of dachs than by loss of sple.

**Competition between polarizing cues**

One revelation from analysis of Pk and Sple localization is that not only can PCP be oriented differently in different places or at different times [62, 101], even at one place, cells can be subject to simultaneous, competing, polarity cues. For example, in the wing, where GFP:Sple localizes differently from GFP:Pk, cells must thus choose between competing polarity cues. Normally, they choose Pk localization cues, because Sple expression is low [59, 60, 99]. Nonetheless, the Sple expressed in the wing is functional and able to direct PCP, as evidenced by the dachs and sple-dependent reversals of hair polarity in pk mutants.

This suggests an interpretation of the relationship between Pk and Sple. Based on observations that pk-sple alleles could have weaker phenotypes than isoform-specific alleles, and that over-expression of Pk or Sple could result in phenotypes reminiscent of sple or pk alleles, respectively, it was proposed that PCP requires a balance between Pk and Sple [57]. However, we suggest that their relationship is better described as a competition. In the wing disc, Pk expression is more abundant than Sple expression, hence Pk "wins", and cells orient in response to cues that are unrelated to Ds-Fat PCP.
When Pk is removed, then Sple can direct PCP, and hair polarity becomes governed by Ds-Fat PCP. Wild-type PCP requires Sple in some places, and Pk in others, but we know of no results that would require a balance between them at anyone place and time.

We further propose that the "competition" between Sple and Pk is carried out by feedback mechanisms that promote polarization. Positive feedback mechanisms, which reinforce the accumulation of co-localized proteins, together with negative feedback mechanisms, which inhibit the accumulation of oppositely localized proteins, are a staple of PCP systems, and have been widely viewed as a means of amplifying, maintaining, and propagating polarization in response to weak polarity signals. The observation that cells sometimes need to choose between competing polarity signals leads us to emphasize that feedback mechanisms could also have a distinct, fundamentally important role in PCP that has not previously been considered - they enable cells to make a discrete choice between competing polarity signals.

**Influence of Ds-Fat signaling on PCP in the wing**

Analysis of wing hair polarity played a central role in development of the hypothesis that Ds-Fat functions as a "global" PCP module and Fz as a "core" PCP module, with polarity guided by the vectors of Fj and Ds expression [61]. However, since Ds-Fat signaling modulates Sple, but not Pk, localization, and Pk, but not Sple, is normally important for wing hair polarity, we infer that Ds-Fat PCP does not normally play a significant role in directing wing hair polarity. Instead, we propose that the hair polarity phenotypes of ds or fat mutants are better understood as a de facto gain-of-
function phenotype, resulting from inappropriate accumulation of Dachs on cell membranes, which then leads to inappropriate localization of Sple, and abnormal polarity. This would also explain how Ds-Fat signaling, stripped of polarizing information, could nonetheless rescue PCP phenotypes: for example, how uniform Ds expression can rescue hair polarity in ds fj mutants [4, 106], and how expression of the intracellular domain of Fat can rescue hair polarity in fat mutants [111], as these manipulations suppress the membrane accumulation of Dachs that would otherwise occur in mutant animals.

More recently, it has been proposed that Ds-Fat PCP provides directional information to orient Fz PCP in the wing by aligning and polarizing apical non-centrosomal microtubules that can traffic Fz and Dsh [59, 65, 67]. While disorganization of these microtubules is observed in fat or ds mutants, we suggest that the inference that Ds-Fat thus orients PCP in wing via these microtubules is misguided. There is evidence both in imaginal discs and in axons that Pk-Sple can orient microtubules [59, 112]. Sple is mis-localized in fat or ds mutant wing discs. Thus, we propose that the effects of ds and fat mutants on microtubules within the wing are likely a consequence of abnormal Sple localization, which disrupts microtubule orientation, but should not be interpreted as evidence for a normal role of Ds-Fat signaling in orienting wing microtubules or Fz PCP.
MATERIALS and METHODS

Drosophila Stocks and Crosses

For investigation of Dachs, Sple or Pk localization we used act> y+>EGFP-dachs /TM6b, act> y+>EGFP-sple /TM6b, or act> y+>EGFP-pk/TM6b [26, 102]. Clones with posterior compartments marked were made by crossing to y w hs-FLP[122];If/CyOGFP; hh-Gal4 UAS-mCD8-RFP /TM6b. Mutant backgrounds examined in clones were ft^{G-rv}/ft^{8}, d^{210}/d^{GC13}, ds^{UA071}/ds^{36D}, d^{GC13} ft^{G-rv}/d^{GC13} ft^{8}, d^{GC13} ds^{UA071}/d^{GC13} ds^{36D}, sple^{l}/sple^{l}, d^{GC13} pk^{30}/d^{GC13} pk^{30}. Flip-out clones were induced by heat shock at 33°C. Additional mutant backgrounds were pk^{30}/pk^{30}, and pk-sple^{14}/pk-sple^{14}. UAS-RNAi-fat (vdrc 9396) or UAS-RNAi-dachs (vdrc 12555) was expressed using nub-Gal4 or C765-Gal4 along with UAS-dcr2.

Immunostaining and fluorescent imaging

Tissues were fixed in 4% paraformaldehyde in PBS followed by permeabilization in PBS with 1% BSA and 0.1% Triton X-100. Primary antibodies used include rat anti-E-cad (1:200, DSHB) and mouse anti-Wg (1:400, DSHB). Secondary antibodies used include Alexa405 and Alexa647 (1:100, life Technologies), GFP and RFP were detected by autofluorescence. Alexa488-phalloidin (1:10, life Technologies) was used to stain hairs in pleura. Protein localization in pleura was determined at ~48h after pupal
formation. Images were captured on a Leica TCS-SP5 confocal or Perkin Elmer Volocity spinning disc confocal.

**Plasmid Constructs and Primers**

Sple was amplified by PCR from pUAST-pk\(^{sple}\) [57] using forward primer (5'-CTCGAACCACGGCGCCGGCCAACATGAGCAGCCTGTCACAACCGGTGGAG-3') and reverse primer (5'-GTGGTTCCAGGGTGACCCGAGATGATGCAGTTCTTGGCTCTTG-3') and cloned using NotI and KpnI sites into pUAST-TM-EGFP:3XFlag [8] after removal of TM-EGFP by NotI / KpnI digestion to generate pUAST-sple:3XFLAG. Sple(N) (first 349 amino acids) was amplified by PCR from pUAST-pk\(^{sple}\) using forward primer (5'ACTCTGAATAGGGAAATTGGGAATTCCAACATGAGCAGCCTGTCACAACCGGTG-3') and reverse primer (5'-GTAGTCGCCTCGAGCCGCGGCCAGCTCATTTGACTCGCTGCGCCG-3') and inserted using InFusion cloning kit into pUAST-app:3XFlag2XStop (gift of B. Staley) after removal of TM-EGFP by EcoRI / NotI digestion, to generate pUAST-sple:3XFLAG. Pk was amplified by PCR from pUAST-pk\(^{pk}\) [57] using forward primer (5'-ACTCTGAATAGGGGAAATTGGGAATTCCAACATGAGCAGCCTGTCACAACCGGTG-3') and reverse primer (5'-GTAGTCGCCTCGAGCCGCGGCCAGCGAGATGATGCAGTTCTTGGCTCTTG-3') and
inserted using InFusion cloning kit into pUAST-app:3XFlag2XStop after by EcoRI / NotI digestion, to generate pUAST-pk:3XFLAG2XStop.

**Co-immunoprecipitation and Western blotting**

Tagged isoforms of Dachs, Ds-ICD, Sple, Sple-N, Pk and GFP (control) were expressed in S2 cells by transient transfection using Effectene of plasmids pUAST-attB-d:V5,His [1], pUAST-TM-DS-ICD:FLAG,V5,His [8], pUAST-attB-sple:3xFLAG, pUAST-attB-sple(N):3xFLAG-2xStop, pUAST-attB-pk:3xFLAG-2xStop, pAc-3XFLAG:GFP [113], pAc-GFP:V5 [10]. Cells were harvested 48 h after transfection and lysed in RIPA (50mM Tris-HCl, pH 8.0; 150mM NaCl; 1% NP-40; 0.5% Sodium deoxycholate; 0.1% SDS; 1mM EDTA; 1mM DTT and 10% glycerol, supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (CalBiochem)). Cell lysates were precleared by incubation in 10µl Protein-A beads for 2 hours at 4°C and later incubated with 10µl anti-V5 beads at 4°C overnight for Co-immunoprecipitation. Anti-V5 beads were washed four times with RIPA, boiled in Laemmli sample buffer at 100°C for five minutes, and run on SDS-PAGE gels. Western blotting was performed using rabbit anti-V5 (1:5000, Bethyl Labs) and mouse anti-Flag (1:3000 Sigma), and Fluorescent conjugated secondary antibodies (Odyssey).
Quantification of polarity

Polarity vectors were determined manually using ImageJ. Rose plots were generated using Matlab. In wing discs, polarity was determined separately in distal, proximal and A-P boundary regions, using Wg and Hh expression as references, with proximal defined as within 5 cells of the fold at the edge of the wing pouch. Cells overlapping the D-V boundary were excluded from analysis. In eye discs, the angle of polarity was estimated relative to the morphogenetic furrow. In the abdomen, the angle of polarity was estimated relative to A-P compartment boundaries.
FIGURES

Figure 1: Localization of Pk and Sple in wing discs, and their interaction with Dachs and Ds

(A) Schematic diagram illustrating the hair polarity (arrows), expression gradients of Ds (magenta) and Fj (cyan) and organization of Ds-Fat and Fz PCP pathway components in the *Drosophila* wing.

(B) Western blots, using antibodies indicated on the right, showing the results of co-immunoprecipitation experiments between V5-tagged Dachs (lanes 1-3,7), Ds-ICD (lanes 4-6,8) or GFP (lanes 9-11) of Flag-tagged Sple (lanes 1,4,9), Sple-N (lanes 2,5,10), Pk (lanes 3,6,11) or GFP (lanes 7,8). Upper panels (Input) show blots on lysates of S2 cells, lower panels (IP-V5) show blots on proteins precipitated from these lysates by anti-V5 beads. Similar results were obtained in three independent biological replicates of this experiment.

(C-G) Portions of wing imaginal discs with clones of cells expressing GFP:Sple (C,E,F) or GFP:Pk (D,G) (green), stained for expression of E-cadherin (blue), and showing either anti-Wg (C-E) or hh-Gal4 *UASmCD8-RFP* (F,G) (red). White arrows indicate direction of polarization of Sple or Pk.
Figure 2: Proteins used in co-immunoprecipitation assays

Schematics of tagged isoforms of full length Dachs, Ds intracellular domain, full length Sple, Sple N-terminal domain and full length Pk.
Figure 3: Ds and Fj gradients in wing discs

(A,B) Expression of Ds (magenta) and fj-lacZ (cyan) in a wild-type wing disc.

(C) Portion of a wing disc with clones of cells expressing Dachs:Cit (green), stained for Wg (red) and E-cadherin (blue). White arrow indicates the direction of Dachs polarization.
Figure 4: Localization of Sple in Ds-Fat pathway mutants

(A-H) Portions of wing imaginal discs with clones of cells expressing GFP:Sple (green) in d\textsuperscript{GCl13}/d\textsuperscript{210} (A and B), ft\textsuperscript{8}/ft\textsuperscript{G-rv} (C), ds\textsuperscript{36D}/ds\textsuperscript{UA071} (D), d\textsuperscript{GCl13}ds\textsuperscript{36D}/d\textsuperscript{GCl13}ds\textsuperscript{UA071} (E and F) and d\textsuperscript{GCl13}ft\textsuperscript{8}/d\textsuperscript{GCl13}ft\textsuperscript{G-rv} (G and H) mutants. Discs were stained for E-cad (blue) and Wg (red). Wg is expressed along the D-V boundary and in proximal rings, the locations of Wg expression shown are indicated. White arrows indicate general direction of Sple polarization.

(I) Rose plots summarizing orientation of Sple or Pk in the indicated genotypes, with proximal at left, distal at right, and in the central row, anterior at top. Orientations were scored separately in the three regions depicted in the cartoon, the number of clones scored is indicated by N.
Figure 5: Pk localization is not affected by loss of dachs, fat or ds

Portions of wing discs with clones of cells expressing GFP:Sple (A,F) or GFP:Pk (B-E) (green) in $d^{210}/d^{GC13}$ (A-C), $ft^{G-rv}/ft^{8}$ (D), $ds^{UA071}/ds^{36D}$ (E) and $d^{GC13} pk^{30}/d^{GC13} pk^{30}$ (F) mutants. Discs were stained for E-cad (blue) and Wg (red) (B, D, E and F) or hh-Gal4 $UAS-mCD8-RFP$ (red) (A,C). The white arrows indicate direction of polarization.
Figure 6: Contribution of Dachs and Sple to PCP mutant wing phenotypes

(A) Cartoons depicting inferred protein localization and hair orientation (brown) in wing cells of the indicated genotypes to explain rescue of \( pk \) by \( dachs \), and rescue of \( fat \) by \( sple \). Faint Sple and Ds indicate lower levels.

(B) Schematic adult wing to show approximate location of panels shown in close-up, as indicated by letters.

(C-N) Close ups of portions of wings (as indicated in panel B) to show hair and bristle orientation in the indicated genotypes. Arrows indicate general direction of polarity. (C-F) Show wing margin bristles, (G-N) show wing hairs, in wild type (C,G), \( pk^{30}/pk^{30} \) (D,H), \( d^{210}/d^{GC13} \) (E,I), \( d^{GC13}/pk^{30}/d^{GC13}pk^{30} \) (F,J), \( UAS-RNAi-fat/+; C765-GaI4/UAS-dcr2 \) (K,M) and \( sple^{1} UAS-RNAi-fat/ sple^{1}; C765-GaI4/UAS-dcr2 \) (L,N).
Figure 7: Hair polarity in wing is not affected by loss of sple or dachs

(A) Schematic adult wing to show approximate location of panels shown in close-up, as indicate by letters.

(B-H) Close ups of portions of wings (as indicated in panel A) to show hair and bristle orientation in the indicated genotypes. Arrows indicate general direction of polarity. Hair polarity in wild type (B,D), sple\textsuperscript{1} mutant (C,E), UAS-dcr2; UAS-RNAi-fat/nub-GaI4 (F), UAS-dcr2; nub-GaI4; UAS-RNAi-dachs (G) and UAS-dcr2; UAS-RNAi-fat/nub-GaI4; UAS-RNAi-dachs (H).
Figure 8: Sple and Pk localization in photoreceptor cells

Localization of GFP:Sple (A-F) or GFP:Pk (G,H) in clones with expression in R4 or R3 photoreceptor cells in wild type (A,G), d^{210}/d^{GC13} (B), ds^{UA071}/ds^{36D} (C), d^{GC13} ds^{UA071}/d^{GC13} ds^{36D} (D), ft^{G-rv}/ft^{8} (E), d^{GC13} ft^{G-rv}/d^{GC13} ft^{8} (F), and sple^{1}/sple^{1} (H) mutants. Rose plots summarize localization based on the indicated number (N) of examples, with equatorial to the right, polar to the left, and anterior (towards the morphogenetic furrow) at top.
**Figure 9: Polarity and gradients in eye discs**

(A) Cartoon illustrating the arrangement and orientation of photoreceptor cells in eye discs at the 5 cell pre-cluster stage.

(B) Schematic illustrating the subcellular localization of Ds-Fat and Fz PCP pathway components at the R4 and R3 interface.

(C,D) Expression of Ds (magenta) and *fj-lacZ* (cyan) in a wild-type eye disc.
Figure 10: Sple and Pk polarity in front of the morphogenetic furrow

(A,B) Portions of eye imaginal discs in front of the morphogenetic furrow with clones of cells expressing GFP:Sple (A) or GFP:Pk (B) (green), stained for expression of E-cadherin (blue). The white arrows indicate general direction of Sple or Pk polarization.
Figure 11: Localization of Dachs, Sple and Pk in abdominal pleura

(A-G) Pleura of wild type (A-C), *ft^{G-rv}/ft^8* (D,E), *d^{GC13} ft^{G-rv}/d^{GC13} ft^8* (F) and *sple'/sple'* (G) pupae with clones of cells expressing of GFP:Dachs (A,D), GFP:Sple (B,E,F) and GFP:Pk (C,G) (green). Posterior compartments are marked by *hh-Gal4 UAS-mCD8-RFP* (red).

(H) Rose plots depicting polarization of GFP:Dachs, GFP:Sple or GFP:Pk in pleural clones of the indicated genotypes; anterior polarization is to the left and posterior polarization is to the right. For wild type and *dachs* mutants, clones were scored separately in A and P compartments. For *fat, ds, fat dachs*, and *ds dachs* the anterior compartment was further subdivided into a front region (A1, anterior-most 8 cells), and the remainder of the A compartment (A*). For *sple*, the A compartment was subdivided into a front region of 5 cells (Af), a back region of 10 cells (Ab), and a middle regions comprising the rest of the compartment (Am); P compartment localization is summarized in Fig S6.
Figure 12: Gradients influencing PCP in the abdomen

Schematic illustrating the orientation of hairs and approximate gradients of Ds, Fj and Hh expression in the abdomen [22, 72].
Figure 13: Influence of Pk and Sple on hair polarity in the abdomen

(A-D) Hair polarity in pleura revealed by F-actin (phalloidin staining) in wild type (A), *pk-sple*<sup>14</sup>/*pk-sple*<sup>14</sup> (B), *pk<sup>30</sup>*/*pk<sup>30</sup> (C) and *sple<sup>1</sup>*/*sple<sup>1</sup> (D) mutants. Yellow asterisk indicates the position of the spiracle, which forms near the center of the anterior compartment. Yellow arrows indicate the region where hair orientation is normal, and red arrows indicate the region where hair orientation is disrupted. Dotted yellow lines mark approximate boundaries between regions with normal and abnormal polarity.

(E-H) Hair polarity in tergites of wild type (E), *pk-sple*<sup>14</sup>/*pk-sple*<sup>14</sup> (F), *pk<sup>30</sup>*/*pk<sup>30</sup> (G) and *sple<sup>1</sup>*/*sple<sup>1</sup> (H) mutant animals. Black arrows indicate regions where hair orientation is normal, and blue arrows indicate the region where hair orientation is abnormal. Dotted blue line mark approximate boundaries between regions with normal and abnormal polarity.
Figure 14: Localization of Dachs, Sple and Pk in abdominal pleura of additional genotypes

(A-J) Pleura of $ds_{UA071}/ds_{36D}$ (A,B,F), $d_{210}/d_{GC13}$ (C,G), $d_{GC13}/ds_{UA071}/d_{GC13}$ $ds_{36D}$ (D,H), $ft_{Grv}/ft^{8}$ (E), $pk^{30}/pk^{30}$ (I) and $d_{GC13}/ft_{Grv}/d_{GC13}/ft^{8}$ (D,J) mutant pupae with clones of cells expressing of GFP:Dachs (A), GFP:Sple (B,C,D,I) and GFP:Pk (E-H,J) (green). Posterior compartments are marked by $hh$-$Gal4$ $UAS$-$mCD8$-$RFP$ (red).

(K) Rose plots depicting polarization of GFP:Dachs, GFP:Sple or GFP:Pk in pleural clones of the indicated genotypes; anterior polarization is to the left and posterior polarization is to the right. Clones were scored separately in A and P compartments.
Figure 15: Influence of Ds-Fat PCP on hair polarity in abdominal pleura

Hair polarity in pleura revealed by F-actin (phalloidin staining) in ft<sup>G-rv</sup>/ft<sup>8</sup> (A), d<sup>2100</sup> / d<sup>GCl13</sup> (B), d<sup>GCl13</sup> ft<sup>G-rv</sup> / d<sup>GCl13</sup> ft<sup>8</sup> (C), ft<sup>G-rv</sup> sple<sup>1</sup> / ft<sup>8</sup> sple<sup>1</sup> (D), ds<sup>UA071</sup> / ds<sup>36D</sup> (E), d<sup>GCl13</sup> ds<sup>UA071</sup> / d<sup>GCl13</sup> ds<sup>36D</sup> (F), ft<sup>G-rv</sup> pk<sup>30</sup> / ft<sup>8</sup> pk<sup>30</sup> (G), ft<sup>G-rv</sup> pk-sple<sup>14</sup> / ft<sup>8</sup> pk-sple<sup>14</sup> (H), d<sup>GCl13</sup> pk<sup>30</sup> / d<sup>GCl13</sup> pk<sup>30</sup> (I) and d<sup>GCl13</sup> sple<sup>1</sup> / d<sup>GCl13</sup> sple<sup>1</sup> (J) mutant animals. Yellow asterisk indicates the position of the spiracle. Yellow arrows indicate the region where hair orientation is normal, and red arrows indicate the region where hair orientation is disrupted. Dotted yellow lines mark approximate boundaries between regions with normal and abnormal polarity.
Figure 16: Influence of Ds-Fat PCP on hair polarity in abdominal tergites

Hair polarity in tergites of $ft^{G-rv}/ft^{8}$ (A), $d^{210}/d^{GC13}$ (B), $d^{GC13} ft^{G-rv}/d^{GC13} ft^{8}$ (C), $ft^{G-rv}/sple^{l}/ft^{8}$ (D), $ds^{UA071}/ds^{36D}$ (E), $d^{GC13} sple^{l}/d^{GC13} sple^{l}$ (F), $d^{GC13} ds^{UA071}/d^{GC13}$ (G), and $d^{GC13} pk^{30}/d^{GC13} pk^{30}$ (H) mutant animals. Black arrows indicate the region where hair orientation is normal, and blue arrows indicate regions where hair orientation is disrupted. Dotted blue line mark approximate boundaries between regions with normal and abnormal polarity.
CHAPTER 5

General discussion
Establishment of planar cell polarity in tissue

The direction of Ds and Fj gradients is important for specifying the direction of PCP in various organs of Drosophila, including wing, eye, leg, notum and abdomen. However, it was not known how cells sense these gradients over a long range in the tissue. We demonstrate that gradients can be measured across the tissue through propagation of Ds-Fat PCP. Upon manipulation of Ds and Fj gradients, Dachs polarity is affected non-autonomously over a range of around 6 cells in wing imaginal disc. This suggests that Ds-Fat polarity can propagate through tissue by coupling of polarity between adjacent cells. The coupling of polarity is achieved through polarized localization of Ds and Fat within a cell, in addition to heterophilic interaction between Ds and Fat among neighboring cells. Moreover, we also demonstrate that Ds can be proteolytically cleaved at two sites to generate two different isoforms and Fat can influence the distribution of these Ds isoforms. Although the functional significance of these isoforms is not known, this identifies a posttranslational influence of Fat on Ds. It has already been reported that Ds can influence phosphorylation of the Fat cytoplasmic domain, which is important for Hippo signaling [10]. The posttranslational influence of Fat and Ds on each other might serve to generate intracellular antagonism between Fat and Ds. This suggests a mechanism to establish the asymmetry of Ds and Fat to opposite sides within a cell. To confirm this hypothesis, it would be essential to know the localization of individual Ds isoforms, and whether Fat can influence this localization. It would also be interesting to study the mechanism that generates the cleaved Ds isoforms and their functional significance in signaling to PCP and Hippo pathway through binding to Fat, as well as through interaction with Riq and Mnb.
The propagation of polarity in tissue has important implications in establishment of PCP robustly throughout the wing. The gradients of Ds and Fj are steep in the proximal region of wing pouch, but are shallow in most of the wing. Hence, local interpretation of gradients would allow establishment of PCP only within a small region in the proximal wing pouch. Through propagation of polarity over a long range, the proximal region could drive the establishment of PCP over a larger area. Based on the extent of Dachs repolarization, the range of propagation would be sufficient to establish PCP only upto around 6 cells. This suggests that polarity might be established at early stage when the wing disc is small enough to encompass the range of polarity propagation (Fig. 1). Propagation of Ds-Fat PCP through coupling of polarity between neighboring cells is also observed in other tissues of Drosophila such as eye disc [25], and might be involved in maintaining polarity in large tissues such as abdominal segments. The abdominal epidermis consists of histoblast cells, which proliferate from histoblast nests during the pupal stages. PCP is established at the stage of histoblast nests and is maintained after the proliferation of histoblast. This is indicated by polarized localization of Dachs in the direction of decreasing Ds expression in the anterior and posterior compartment of abdominal segments. Thus, propagation of polarity may serve as a robust mechanism to align PCP over very large tissues, even in mammalian organs such as skin. Indeed, gradients of Ds-Fat pathway proteins in vertebrates are reported to influence PCP in some organs. Dchs1 and Fat4 are expressed in opposing gradients in hindbrain of mouse and influence the migration of facial branchiomotor neurons [34]. Fjx1 and Dchs1 have complementary expression pattern in other organs like kidney, lung, and intestine. Hence, it is possible that these proteins are expressed in shallow gradients in large organs
such as skin and PCP is coordinated across the tissues through propagation of polarity. However, asymmetric localization of Dchs1 and Fat4 within a cell is not yet reported in vertebrates. Hence, it would be interesting to study if the mechanisms of PCP propagation are conserved in vertebrates.

**Influence of Fat activity on Dachs localization**

Fat controls growth and PCP through regulating Dachs localization. Dachs and Ds interact with Sple to influence PCP, and Dachs interacts with Zyxin to influence Wts levels and influence Hippo pathway. Mutation in dachs can suppress fat mutant overgrowth phenotype and partially suppress fat mutant PCP phenotype. However, the importance of membrane localization of Dachs in Ds-Fat signaling was not clear. We demonstrate that targeting Dachs to all the sides of the membrane independent of Fat activity resulted in strong activation of Hippo signaling and loss of PCP, as indicated by the overgrowth of wings, upregulation of Yki target gene ex, misorientation of hair PCP and shortened cross-vein distance. Thus, Dachs membrane localization is important for regulation of both Hippo signaling and PCP through Fat. In vertebrates, Fat4 is known to influence PCP whereas Fat1 influences Hippo pathway, but Dachs homolog is not yet identified [35, 114]. However, it remains possible that other myosins might function similar to Dachs to regulate PCP. Indeed, Dachs is closely related to myosin X and mutation in myosin X results in PCP defects such as misorientation of the mitotic spindle, suggesting that Fat4 may interact with myosin X to regulate PCP in vertebrates [115].
Hence, it would be interesting to study if myosin X localization is important for PCP regulation by Fat4.

Fat activity correlates with Dachs localization. In absence of Fat, Dachs is present on all the sides of the membrane, whereas upon ectopic expression of Fat, Dachs is pushed off into cytoplasm. However, the mechanism by which Fat influences the membrane localization of Dachs is still not completely clear. Dachs membrane localization is dynamic, as indicated by the lateral movement of GFP-tagged Dachs puncta on membrane and upto 60% recovery after photobleaching during FRAP analysis. Moreover, Fat influences the mobility of Dachs such that in absence of Fat, the recovery after photobleaching was significantly slower than wild type conditions. This suggests that activated Fat may tend to mobilize Dachs such that Dachs accumulates preferentially on the distal side, since Fat is polarized towards the proximal side. Moreover, Dachs puncta colocalize with Ds puncta on distal side and Dachs can physically interact with the intracellular domain of Ds. This might contribute to stabilizing Dachs on distal side of the cell. We did not observe any significant change in Dachs membrane dynamics upon RNAi-mediated knockdown of Ds, which might be due to insufficient knockdown of Ds levels. Also, absence of Fat decreased the stability of Dachs on membrane. Although this might suggest that activated Fat would tend to stabilize Dachs on the membrane, it might also be an indirect effect due to activation of Hippo pathway. It is possible that there are additional proteins involved in regulation of Dachs membrane localization, which are expressed downstream of Hippo pathway. Moreover, the role of Dachs accumulation in puncta is not known. It is possible that these puncta might be involved in trafficking of Dachs to the membrane and Fat might influence this trafficking. Indeed, endocytosis and
vesicle recycling is involved in maintaining localization of membrane-associated proteins [116]. Recently, it has been reported that the ubiquitin ligase Fbxl7 interacts with Fat to regulate the levels and localization of Dachs [18, 19]. However, it was not clear whether Dachs is the direct target for ubiquitination by Fbxl7. Overall, these observations suggest the presence of additional components downstream of Fat and upstream of Dachs that relay the signal from Fat to Dachs. Identifying these components would be essential in understanding the regulation of PCP by Ds-Fat pathway.

**Crosstalk between PCP pathways**

The gradients of Ds and Fj align the polarity of Ds-Fat system along the axes of the tissue. Ds and Fat are asymmetrically localized in a cell under the influence of these gradients, ultimately resulting in polarization of Dachs along the direction of these gradients. However, it was not clear how the Ds-Fat pathway influences PCP downstream of Dachs. Also, it was not clear how the Ds-Fat pathway influences the Fz pathway to align its polarity along the tissue axes. We demonstrate that Dachs and Ds can independently interact with and influence the localization of Sple in various tissues of *Drosophila*. The requirement for Dachs and Ds varies for different regions within an organ, and between various organs. Dachs is necessary for Sple localization in distal region of wing. However, in proximal wing and eye, Dachs is dispensable but Ds is required for Sple localization. It would be interesting to study if such variation in requirement for Dachs and Ds exists in other organs where Ds-Fj gradients regulate PCP,
such as notum and tarsal segments of the leg. Overall, Dachs-Sple and Ds-Sple interactions provide a molecular mechanism to mediate the connection between the two PCP pathways. The nature of crosstalk between PCP pathways in vertebrates is not clear. Genetic interaction between Fat4 and Vangl2 has been observed in mice, where some of the Fat4 mutant phenotypes are enhanced by Vangl2 mutation [36]. However, Sple isoform is not conserved in vertebrates. Also, vertebrate homolog of Dachs is not yet identified, although other myosins may function similar to Dachs. Hence, Fat4-Dchs1 might influence PCP through other mechanisms.

Pk and Sple isoforms can localize to the opposite sides within a cell, suggesting that they can respond to different upstream localizing cues. Indeed, it has been reported that several cues including Hh, Wg and Notch can orient Pk polarization in wing. In absence of localization cues from Ds-Fat pathway, Sple can respond to the same localizing cues as Pk due to shared motifs. Moreover, PCP activity is mediated through the C-terminal region common to Pk and Sple. Overall, these observations suggest that cells are subjected to competing polarizing cues from Pk and Sple, and they choose the cue based on protein abundance. Thus, Pk and Sple function as sensors of distinct upstream polarizing signals and ultimately bring about robust polarization within a cell (Fig. 2). This mechanism might have evolved to integrate information from various global polarizing cues with varying strength and directions, and interpret these signals in a way such that PCP consistently points in one direction. That said, it is still not clear how many global polarizing cues exist. Mutations in pk-sple locus that result in loss of both Pk and Sple isoforms have mild polarity phenotypes in wing and leg, and polarity is
normal in large areas of abdomen. These observations suggest the presence of multiple global polarizing cues and PCP systems to interpret these signals.
Figures

Figure 1. Establishment of planar cell polarity in wing

Model comparing the establishment of PCP in case of local sensing and long range sensing of gradients. Local sensing would be sufficient to establish PCP only in the proximal region of wing pouch (indicated by green color), where the gradients are steep. Long range sensing would allow establishment of PCP throughout the wing pouch of early third instar wing disc through propagation of PCP from the proximal region.
Establishment of polarity

Early 3\textsuperscript{rd} instar wing disc

Local gradient sensing

Polarity established only in proximal region

Late 3\textsuperscript{rd} instar wing disc

Long range gradient sensing

Polarity established in whole wing pouch

Ds gradient
Region where polarity established
Figure 2. Signal integration through Pk and Sple isoforms

Model for interpreting the upstream polarizing cues in Sple and Pk-dominant tissues.

Sple polarity is regulated by Ds-Fat pathway. Hence, PCP in Sple-dominant tissues would be majorly influenced by Ds-Fat pathway. Pk polarity is regulated by Hh, Wg and Notch pathways. Hence, these pathways would have major influence on PCP in Pk-dominant tissues.
Signal integration through Pk and Sple isoforms

**Sple tissue**
- Ds-Fat pathway
- Hh, Wg and Notch pathway
  - Sple
  - Pk
  - Planar cell polarity

**Pk tissue**
- Ds-Fat pathway
- Hh, Wg and Notch pathway
  - Sple
  - Pk
  - Planar cell polarity


